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Understanding the Effect of BVDV on Antigen Presenting Cells and Cytoplasmic Trafficking

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UNDERSTANDING THE EFFECT OF BVDV ON ANTIGEN PRESENTING CELLS AND CYTOPLASMIC TRAFFICKING

By

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This dissertation is approved as creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

%: Percentage

°C: Degree centigrade

µg: Microgram

µl: Microliter

3-MA: 3-methyladenine

APC: Antigen presenting cells

Atg: Autophagy-related gene

ATP: Adenosine triphosphate

B-cell: B lymphocyte

BCG: Bacille Calmette-Guerin

BD: Becton Dickinson

BHV-1: Bovine herpes virus one

BM: Bone Marrow

BSA: Bovine serum albumin

Bt cell: Bovine turbinate cells

BVD: Bovine viral diarrhea

BVDV: Bovine Viral Diarrhea Viruses

C: BVDV nucleocapsid protein

CARDs: Caspase recruitment domains

Caspase-2: Cystiene-containing aspartic acid-specific protease-2

CCR5: Chemokine receptor type 5

CCR7: Chemokine receptor type 7

CD117: Cluster of differentiation 117

CD14: Cluster of differentiation 14

CD2: Cluster of differentiation 2

CD21: Cluster of differentiation 21

CD28: Cluster of differentiation 28

CD2L: Cluster of differentiation 2 ligand

CD3: Cluster of differentiation 3

CD34: Cluster of differentiation 34

CD4: Cluster of differentiation 4

CD40: Cluster of differentiation 40

CD8: Cluster of differentiation 8

CD80: Cluster of differentiation 80

CD86: Cluster of differentiation 86

CLIP: Class II-associated invariant chain peptide

CLP; Common lymphoid progenitor

CMI: Cell mediated immunity

CMP; Common myeloid progenitor

CO2: Carbon dioxide

Cp: Cytopathic

CPE: Cytopathogenic effect

CTL: Cytotoxic T lymphocyte

CTLA4: Cytotoxic T-Lymphocyte Antigen 4

DAPI: 4,6-diamidino-2-phenylindole

DEC205: A type I cell surface protein expressed primarily by dendritic cells

DENV: Dengue virus

Ds RNA: Double stranded ribonucleic acid

DTH: Delayed type hypersensitivity

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

FBS: Fetal Bovine Serum

FCS: Fetal calf serum

FITC: Fluorescein isothiocyanate

GFP: Green fluorescent protein

GMCSF: Granulocyte/macrophage colony-stimulating factor

Gp: Glycoprotein

HCMV: Human cytomegalovirus

HCV: Hepatitis C virus

HIV: Human immunodeficiency virus

hr: Hour

IACUC: Institutional Animal Care and Use Committees

ICAM-1: Intercellular Adhesion Molecule 1

IFA: Indirect fluorescence assay

IFNά: Interferon alpha

IFNγ: Interferon gamma

IgG: Immunoglobulin G

IgG: Immunoglobulin M

IHC: Immunohistochemistry

IL: Interleukin

IRFs: Interferon regulatory factors

Kd: Kilodalton

(LANA1: Latency-associated nuclear antigen1

LC3: Light chain 3

LFA-1: Lymphocyte function-associated antigen 1

Lin: Lineage

Log: Logrithum

LPS: Lipopolysaccharide

MAP: *Mycobacterium avium* subspecies paratuberculosis

MARCH1: Membrane-Associated RING-CH Protein

Mb: Millions of base pairs

M-CSF: Macrophage colony stimulating factor

MDBK: Madin Darby bovine kidney cells

MDDC: Monocyte-derived dendritic cells

MDM: Monocyte-derived macrophages

MEM: Minimal essential medium

MFI: Mean fluorescent intensity

mg: Milligram

MHC I: Major histocompatibility class I

MHC II: Major histocompatibility class II

min: Minute

ml: Milliliter

MLV: Modified live vaccine

MOI: Multiplicity of infection

mTOR:Mammalian target of rapamycin

NCP: Non cytopathic

Ng: Nano gram

NS: Non structural protein

OD: Optical density

PI: Persistent infection

PAMP: Pathogen associated molecular pattern

PBMC: Peripheral mononuclear cells

PBS: Phosphate buffered saline

PCD: Programmed cell death

PD-1: Programmed Death 1

PD-1L: Programmed Death 1 ligand

PFA: Paraformaldehyde

PFU: Plaque-forming unit

p.i: Post infection

PI: Persistent infection

PKR: Protein kinase R

PMNC: Peripheral morphonuclear cell

PPD: Purified protein derivative

PRR: Pathogen recognition receptors

P-SMAC: Peripheral supramolecular activation cluster

q-PCR: Quantitative polymerase chain reaction

RIG-I: Retinoic acid-inducible gene I

RNA: Ribonucleic acid

RPMI: Roswell Park Memorial Institute (Medium)

RT-PCR: Reverse transcriptasepolymerase chain reaction

TAP: Transporter associated with Antigen Processing

TICAM-1: Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule

T cell: T lymphocyte

TCR: T cell receptor

TGAC: Tifton Georgia cytopathic Bovine Viral Diarrhea Viruse

TGAN: Tifton Georgia non cytopathic Bovine Viral Diarrhea Viruses

TGF: Tumor growth factor

Th1: T helper one

Th2: T helper two

TLR: Toll like receptor

TNF: Tumor necrosis factor

TSLP: Thymic stromal lymphopoietin

U: UNIT

UTR: Untranslated region

UV: Ultraviolet

VMRD: Veterinary Medical Research & Development

WNV: West Nile virus

μl: Microliter

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ABSTRACT

UNDERSTANDING THE EFFECT OF BVDV ON ANTIGEN PRESENTING CELLS AND CYTOPLASMIC TRAFFICKING

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2013

Bovine viral diarrhea viruse (BVDV) causes immunosuppression and persistent infection that are great threats to the health of cattle. Antigen presenting cells (APC), like dendritic cells (DC), provide active surveillance and present antigens to the immune system. Monocyte-derived dendrite cells (MDDC) were used as an *in vitro* model to evaluate the effect of BVDV on DC cell surface markers expression and the role of DC in BVDV dissemination. The study used 4 different strains of BVDV: severe acute noncytopathic (ncp) BVDV2a-1373, mild acute ncpBVDV2a-28508-5, and type1b mucosal disease virus pair, cytopathic (cp) TGAC or ncp TGAN strains.

MDDC did not produce infectious virus. However viral RNA was replicated and translated into the NS5a viral protein. The MDDC precursor, monocytes were infected and produced infectious BVDV. The infectious BVDV production was reduced over time as the monocyte differentiated to MDDC and virus production was completely lost by 120 hr of differentiation. The cp-BVDV1b-TGAC up regulated the MHCI, MHCII and CD86 expression while the three ncp BVDV strains reduced the MHCI, MHCII and CD86 expression in MDDC.

The role of BVDV infection in autophagy induction and effect of autophagy in BVDV replication was examined. BVDV infection induced autophagy in MDBK cells

and Bt cells. There was no significant difference between cp or ncp strains of BVDV in autophagosome formation. The autophagy inducing drug, rapamycin, enhanced the viral replication while the autophagy inhibiting drug, 3MA (3-Methyladenine), suppressed viral replication. The co-localization study, using BVDV NS5A or E1 with GFP-LC3 revealed that BVDV did not replicate in autophagosomes.

The effect of cp or ncp BVDV infection *in vivo* on immune polarization to T helper-2 (TH2) or T helper-1 (TH1) by measuring serum IgG1 and IgG2 concentrations was done using the TGAC-TGAN virus pair. Ncp TGAN directed the immune response toward a TH1 or cellular immune response while the TGAC cytopathic strain of BVDV directed the immune response toward TH2 or humoral immune response. The cumulative findings of these studies contributed to the general body of knowledge regarding immunosuppression associated with BVDV infections.

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CHAPTER 1.

RESEARCH OBJECTIVES AND LITERATURE REVIEW

INTRODUCTION

Infections with bovine viral diarrhea viruses (BVDV) are a major problem for the cattle industry. BVDV causes a wide variety of respiratory, gastrointestinal and reproductive syndromes. Dendritic cells (DC) are important antigen presenting cells that play a crucial role in antigen monitoring, processing and presentation. Because of their important role of DC in activating the acquired immune system, BVDV infection of DC may have a devastating impact on the immune system and virus dissemination in body.

Host immunity has two components: innate and adaptive immune response. The innate immune response is quicker but is not pathogen-specific and has no immunological memory. The adaptive immune response is slower but is pathogen-specific with long lasting immunological memory. When the adaptive immune system encounters a pathogen for the second time, it produces a rapid and more effective response against that pathogen. DC play a critical role in connecting the innate and adaptive immune response through antigen presentation and naive T cell activation.

BVDV infects a wide variety of cell types and has a predilection for cells of the immune system including antigen-presenting cells like monocytes that develop into DC. The viral infection of DC may interfere with its surface markers expression, antigen presentation and T-cell activation. Interference in DC activity may be one of the causes of immunosuppression associated with BVDV infections. The impairment of DC function may also result in evasion from the immune response. The major objective of

this study was to investigate the effect of BVDV infection on monocyte-derived dendritic cell (MDDC).

The hypotheses were: 1) MDDC are targets of BVDV production resulting in dissemination of BVDV to secondary lymphoid tissues and other tissues; 2) Infection of monocyte-derived dendritic cell (MDDC) with BVDV causes changes in the phenotype of MDDC (cell surface molecules expression); 3) BVDV infection induces autophagy and uses autophagosome for its replication and productive infection and 4) *In vivo* antibody response against cytopathic BVDV polarizes the T cell response to TH2 response while the antibody response to noncytopathic BVDV is polarized to TH1 response

RESEARCH OBJECTIVES

1) **To generate bovine monocyte-derived dendritic cells (MDDC) for use as an *in vitro* model for studying host cell-virus interaction.** Under this objective three approaches were taken:

a) Optimize a previously described method for separation of monocytes from bovine peripheral blood that yields a high number of viable monocytes.

b) Optimize culture conditions for maturation of monocytes to MDDC.

c) Characterize morphological and phenotypic characteristics of MDDC.

2) **Investigate the effect of BVDV on MDDC viability and BVDV replication and virus production in MDDC**

The three approaches were:

a) Study the effect of BVDV biotypes and virulence on MDDC viability through trypan blue exclusion assay

b) Investigate the ability of BVDV to replicate in MDDC

c) Investigate BVDV virus production in MDDC.

3**) Investigate the effect of BVDV on MDDC phenotypes**

Under this objective, one approach was taken:

a) Study the cell surface marker expression (MHC I, MHC II and CD86) in MDDC following BVDV exposure through flow cytometry analysis

4) **Effect of BVDV infection on autophagosome induction in infected cells.**

The three approaches were:

a) Determine the effect of BVDV strains on autophagosome formation.

b) Determine the relationship between autophagosome formation and virus production

c) Determine the interrelation between viral replication and autophagosome formation through co-localization study of viral proteins with autophagosome marker (LC3).

5) **Effect of BVDV strains on polarity of immune response.**

Two approaches were taken:

a) Determine the effect of cytopathic and non cytopathic stain of BVDV on total serum IgG levels in calves.

b) Determining ratio of IgG1 to IgG2 in serum profile after infection of cytopathic or noncytopathic stain of BVDV to determine TH2 and TH1 immune response respectably.

LITERATURE REVIEW

1.1 BVDV Associated Disease:

Bovine viral diarrhea viruses (BVDV) are viral pathogens that are the source of several different diseases and syndromes that impact the cattle industry. Despite 60 years of vaccination, BVDV infections remain a source of significant economic loss for producers in the United States **(Ridpath, 2012)** and world **(Stahl and Alenius, 2012**). The evidenced by outbreaks of hemorrhagic syndrome and severe acute bovine viral diarrhea beginning in the 1980s and 1990s **(Goens, 2002)**. The International Committee on Virology Taxonomy recognizes two species of BVDV, BVDV1 and BVDV2 (ICTV, 2013).There are significant antigenic and genomic differences between isolates from the two species **(Ridpath et al., 2003).** In addition, some BVDV2 strain cause a severe clinical disease, known as hemorrhagic syndrome, that has not been observed following infection with BVDV1 **(Corapi et al., 1989; Carman et al., 1998; Ridpath, 2003).** Viruses from both BVDV species are further classified into two biotypes: cytopathic (cp) BVDV and noncytopathic (ncp) BVDV. This distinction is based on morphological changes in cultured epithelial cells following infection **(Gillispie et al., 1962).**

BVDV is capable of a wide spectrum of disease and clinical symptoms. The clinical presentation of disease can range from clinically mild to severity

form of disease depending on the virulence of the strain and health state of the infected host. It may be clinically mild acute, clinically severe acute, abortion, fetal death, congenital defects, persistent infection and mucosal disease **(Pritchard, 1963; Brownlie, 1990; Moennig and Liess, 1995; Peterhans et al., 2010).** Acute infection is also known as primary infection or transient infection. Typically, cattle develop an immune response against BVDV that effectively clears the virus during the course of acute infection. Acute infections can be caused by either biotypes of BVDV. Clinical signs of acute BVDV infection may include fever, diarrhea, general respiratory sysmptoms, inappetance, depression, lymphopenia, and thrombocytopenia with decreased milk productionand nasal discharge **(Carman et al., 1998; Baule et al., 2001).** Acute infection of BVDV in cattle plays an important role as an immunosuppressive agent or act as a potentiator for other diseases **(Houe, 1995; Potgieter, 1995; Taylor et al., 1997)**. In typical acute infections, virus is generally found in lymphoid-associated tissues as well as intestinal mucosa **(David et al., 1993; Hibberd et al., 1993)**. In rare cases, acute infections may progress to highly severe presentation known as severe acute of periacute BVDV. Periacute BVDV is associated with high virulence strains of BVDV. The clinical manifestation of severe acute infection include fever, pneumonia, diarrhea, and sudden death occurring in all age groups of cattle. Gross lesions in the alimentary tract are similar to those associated with mucosal disease. Some animals develop severe thrombocytopenia with hemorrhages (hemorraghic syndrome) **(Bolin and Ridpath, 1992; David et al.,**

1994; Carman et al., 1998). The chronic BVDV infection can be established when BVDV maintained itself in immunoprivileged sites such as ovarian tissue, testicular tissue and central nervous system tissue, and circulating white blood cells. Infections of circulating white blood cells may change their ability to stimulate adaptive immune response and facilitate chronic infection of these cells **(Givens and Marley, 2013).**

Following implantation, transplacental infection of the developing fetus can occur from the dam with either biotype of BVDV **(Fredriksen et al., 1999).** The outcome of the infection is largely dependent on the timing of the infection, the immunocompetence of the developing fetus, the virus biotype involved, and the virulence of the virus **(Grooms, 2004)**. During pregnancy, the ncp BVDV strains generally infect the trophobloast cells of the placental and infect the growing fetus by crossing placental barrier **(Fredriksen et al., 1999**). BVDV infection during pregnancy may result in early embryonic death, abortion, congenital defects, immunotolerance and birth of weak calves **(Grooms, 2004).** The congenital defect in calves may include brachygnathism, growth retardation, malformations of the brain and cranium, and rare extracranial skeletal malformations **(Blanchard et al., 2010).** However, if ncp BVDV infects bovine fetuses during the first 40-120 days of pregnancy persistently infected (PI) calves may be the result **(Chase et al., 2004).** PI calves are immunotolerant and remain a source of infection to other animals. Superinfection of PI animals with either an antigenically homologous or a vaccine cpBVDV strain may results in mucosal

disease, a fatal disease syndrome **(Brownlie et al., 1984).** Mucosal disease is characterized by high mortality and extensive lesions in gastrointestinal tract **(Nettleton and Entrican 1995).**

1.2 Bovine Viral Diarrhea Virus:

Bovine viral diarrhea virus (BVDV) is a member of the Pestivirus genus and the family Flaviviridae **(Handelet al., 2011)**. It is a single-stranded, positive-sense RNA virus. BVDV has a genome of approximately 12.5 kb with single open reading frame. The genome translates a single polyprotein that is cleaved into individual viral proteins by host cell and viral proteases (Figure 1-1). The C protein functions to package the genomic RNA and provide structure for the virion envelope. The other structural proteins include three envelope-associated glycoproteins, Erns (gp48), E1 (gp25), and E2 (gp53) that are cleaved by cellular proteases (Figure 1-1). The Erns, E1, and E2 have important roles in virus binding and cell entry as well as for immunologic recognition by the host. The E2 protein contains the major recognition sites for BVDV neutralizing antibodies against BVDV. The neutralizing epitopes of E2 are important targets for BVDV vaccine efficacy to induce effective humoral immune response against BVDV **(Donofrio et al., 2006; Chimeno Zothet al., 2007).** Unlike E1 and E2, Erns is dispensable for cellular entry **(Iqbal et al., 2004; Ronecker et al., 2008).** The E1 protein is predicted to have various functions such as a membrane anchor for E2. The E1 and E2 form E1-E2 heterodimers. The E1-E2 heterodimers appears to be essential for cell entry of BVDV **(Rumenapf et al., 1993; Ronecker et al., 2008).** Among the six nonstructural proteins in the noncytopathic BVDV genome, the Npro (p20) is the first protein produced from the

open reading frame (Figure 1-1). It has papain-like protease activities. The next nonstructural protein produced is NS23 (p125) (Figure 1-1). This protein has several unique characteristics that suggest its involvement in multiple functions like, a protease, and a helicase. In cytopathic BVDV strains, the NS23 protein cleaves into two proteins: NS2 (p54), and NS3 (p80) (Figure 1-1). The NS3 protein, which is unique to the cytopathic BVDV biotype contains the protease and helicase activity of the NS23 protein. Other nonstructural proteins include NS4A (p10), NS4B (p32), NS5A (p58) and NS5B (p75) (Figure 1-1). The NS5B (p75) acts as RNA-dependent-RNA polymerase and is needed to replicate the viral genome **(Elbers et al., 1996)**. The virus replicates in the cytoplasm and is released by budding **(Grummer et al., 2001)**. The NS5A protein of HCV (hepatitis C virus), other virus of family Flaviviridae has key roles in viral RNA replication and modulation of the physiology of the host cell. The NS5A protein of HCV modulate the cellular environment that favor the virus replication and initiate the persistence HCV infection **(Macdonald and Harris, 2004)**.

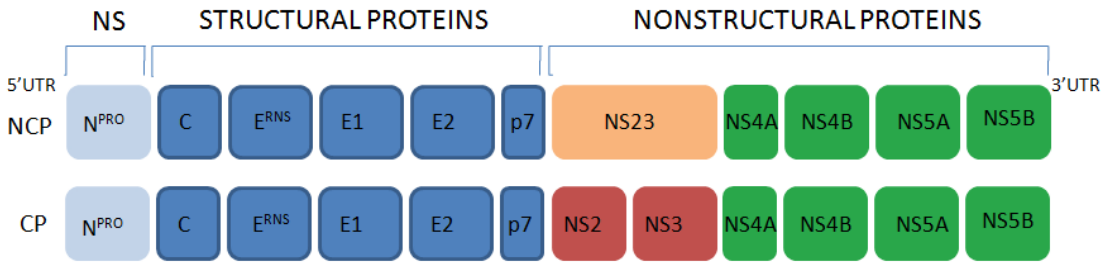

Figure 1-1 The schematic representation of the BVDV encoded proteins.

BVDV is a single-stranded RNA virus comprised of 12,300 nucleotides. BVDV consists of a single open reading frame between two untranslated regions (5' and 3'). The poly protein encoded is autocleaved into single proteins both structural and non-structural. In cytopathic biotype viruses NS23 protein cleave in to NS2 and NS3 proteins (lower panel) while in noncytopathic biotype viruses NS23 does not cleave (upper panel). from: Morarie, S.E. (2012). "*Unraveling the biology of bovine viral diarrhea virus (bvdv) persistent infections: integrating field and laboratory studies."* Ph.D. Thesis. South Dakota State University, Brookings, SD 57007, U.S.A (Pt): 9.

1.3 BVDV-induced Immunosuppression:

An acute BVDV infection results in transient immunosuppression. BVDV infection affects both adaptive and innate immune system. The granulocytes and monocytes of the innate immune system are equally susceptible to BVDV with reduction in numbers and inhibited functions observed in both the populations **(Brewoo et al., 2007).** BVDV affects thymic and follicular T-lymphocytes, as well as follicular B-lymphocytes resulting in severe reduction of circulating lymphocytes number and decreased functions.

BVDV infects almost all types of bovine cells including antigen-presenting cells (APC). The APC infected with ncp BVDV isolates does not result in induction of type I interferons (1FNF) *in vitro* **(Diderholm and Dinter 1966; Adler et al., 1997)** and to blocks the induction of type 1 IFN by double-stranded RNA (dsRNA) or infection with other viruses **(Schweizer and Peterhans, 2001)**.

An acute BVDV infection or vaccination with MLV BVDV results in decreased delayed type hypersensitivity (DTH) to *Mycobacterium avium* subspecies. DTH responses to *Mycobacterium paratuberculosis* purified protein derivative (PPD) were decreased in cows experimentally exposed to *M. paratuberculosis* 7 days vaccination with a modified-live bovine viral diarrhea virus (ML-BVDV) vaccine **(Thoen and Waite, 1990)**. Acute infections of cattle with non-cytopathic BVDV temporarily compromises diagnostic tests for *M. bovis* infections and result in a failure to identify cattle with tuberculosis. MLV BVDV vaccination inhibited neutrophil mediated antibody-dependant cellular cytotoxicity **(Roth and Kaeberle, 1983).** Further, BVDV-infected monocytes have marked reductions in leukotriene B4; leukotriene B4 is an important mediator in IL-1, IL-2 and IFN-alpha production **(Atluru et al., 1992).**

Protective immunity against intracellular pathogens requires a strong TH1 immune response. TH1 immune response is predominantly characterized by the production of IFN-γ **(Abbas et al., 1996; O'Garra, 1998)** and IL-12 **(Trinchieri, 1995; Kalinski et al., 1999)**. Type I IFN and IL-12 gene expression were significantly up regulated in monocytes within 1 hr p.i. with ncp BVDV but not cp BVDV. The differences between Type I IFN and IL-12 were not observed between cp and ncp BVDV

after 24 hours p.i. Both BVDV biotypes also suppressed pro-inflammatory cytokines including TNF-alpha, IL-1 beta, IL-6, and co-stimulatory molecules CD80 and CD86 24hr p.i. **(Lee et al., 2008).**

1.4 Effect of BVDV on humoral Immune Response:

Passively acquired antibodies against BVDV confer immunity to newborn calves **(Ridpath et al., 2003).** The amount of passive immunoglobulin transferred from dam to newborns vary with the dam's condition and age. Calves born to younger dams tend to have have lower immunoglobulin levels, resulting from passive transfer, than calves that were born to older cows **(Perino et al., 1995).** The presence of passive antibodies at the time of vaccination can inhibit the development of the specific antibodies. The half-life of maternally-derived BVDV antibody is between 20 and 23 days. For calves, the amount of time required for maternal antibody decay to reach seronegative status for BVDV 1 ranged between 118 and 192 days and for BVDV 2 between 94 and 158 days **(Fulton et al., 2004)**. In the absence of passive antibodies, the primary antibody response to BVDV challenge develops within one to three weeks post-vaccination. Neutralizing serum antibodies against BVDV were monitored for three years in 35 cattle that were infected with live modified virus as calves. Twenty-four (24) of the calves were inoculated intramuscularly or intranasally while 11 contracted the infection naturally. All the experimentally infected calves seroconverted within 14 to 28 days after inoculation, and all the animals had the detectableserum antibodies to BVDV, three years after infection **(Fredriksen et al., 1999)**. Serum antibodies have the ability to protect against viral infection. The protection provided from the vaccine response dependents on the strain of

the virus administered along with the level and the isotype of antibodies produced **(Chase et al., 2004)**and adjuvant used in the vaccine **(Leroux-Roels, 2010).** Both cytopathic and non-cytopathic BVDV biotypes can be neutralized by IgG1 and IgG2 in absence of complement **(Howard, 1990)**. A study conducted with 14 type 1 and 6 type 2 BVDV strains indicated that both genotypse produced similar amounts of neutralizing antibody against respective strains in calves **(Fulton et al., 1997).** Neutralizing antibody provided protection against BVDV infection and reduced the BVDV shedding from infected animals **(Howard et al., 1989)**. BVDV infection reduced circulating B and T cells in cp BVDV-infected cattle **(Bolin et al., 1985).** The incubation of bovine splenic lymphoid cells with ncp bovine viral diarrhea virus (BVDV) for 5 days inhibited the development of plasma cell with reduced synthesis of IgG or IgM **(Atluru et al., 1979)**.

1.5 The Effect of BVDV on Cell Surface Molecules Important for Antigen Presentation:

The major histocompatibility complex (MHC) was originally discovered as transplantation antigens that determine the compatibility of tissues between different individuals. The complex is comprised of three classes of genes. Class I and II genes encode molecules that are expressed on cell surface and class III genes was discovered that encode several components of the complement system. Later studies with inbred strains of mice showed that class I and class II genes play an important role in controlling both humeral and cell-mediated immune responses. The MHCI molecules are used as context by cytotoxic T-lymphocytes and MHCII are used as context by helper and other regulatory T cells **(Klein and Figueroa, 1986)**. In humans, the major histocompatibility

complex (MHC) contains at least 128 functional genes in human of which, more than 20% of which have functions in immunity..

The MHC molecule in cattle is known as the bovine leucocyte antigen complex (BoLA) **(Untalan et al., 2007).** The BoLA spans approximately 2.6 megabase pairs (Mb) of the cattle genome while the MHC in human and mice is 4 Mb and 1.5 Mb respectively **(Lewin, 1996; Rothschild et al., 2000)**. In BoLA, the genes are organized into three distinct classes (class I, II, and III) similar to human and mice. In BoLA, each of these classes is divided into regions and sub-regions, containing genes and pseudo genes **(Andersson and Davis, 2004)**. In cattle, BoLA genes are located on chromosome 23 **(Fries et al., 1986; Fries et al., 1993)** while in human and mice MHC genes are located at chromosome 6 and chromosome 17 respectively **(Lewin, 1996)**. The structure and organization of the MHC genes of cattle is similar to human and mice with few differences. The major difference between the organizations of the BoLA of cattle and MHC molecules of human and mice is that the BoLA complex is found in two separate regions of the chromosome rather than a single cluster of genes. The larger gene cluster is located at BTA **(**Bos taurus autosome**)** 23 band 22 and apparently contains all of the bovine class I and class III sequences, and genes encoding both subunits of the classical class II proteins DQ and DR. The DQ and DR regions are tightly linked to other BoLA genes, including HSP70, CYP21, and several class I loci **(Bishop et al., 1994)**. The remaining BoLA class II loci (DIB, DNA, DOB, DYA, DYB, TCP1, LMP2, LMP7 and TAP2) are located in a cluster near the centromere at BTA 23 band 12-13 **(Rothschild et al., 2000).** The class I region contains at least 10 class I genes and pseudogenes

(Lindberg and Andersson 1988; Bensaid et al., 1991) and at least four class I loci are transcribed in cattle **(Ellis et al., 1999)**. The BoLA class I genes are polymorphic and 28 distinct BoLA class I sequences have been identified (**Takeshima and Aida, 2006)**. The BoLA genes I and II are associated with genetic resistance and susceptibility to a wide array of diseases and other traits such as milk yield, growth and reproduction **(Weigel et al., 1990; Rupp et al., 2007; Untalan et al., 2007).**

In many vertebrate species, including cattle the MHC class I and class II loci exhibit an extraordinarily high degree of polymorphism, particularly in exon 2 of the beta genes. This variation is probably maintained through selection involving interactions of the immune system and the pathogens **(Parham and Ohta, 1996)**. MHC II molecules are primarily expressed in antigen presenting cells (APC). These cells include B-cells, macrophages and dendritic cells. MHCII can be induced in epithelia cells using various cytokines like TNF alpha and IFN gamma **(Helbig et al., 1990; Makhoul et al., 2012).**

The activation of naïve T cells requires several steps. The first step is the danger signal or signal zero. The danger signal plays an important role in T cell communication to APC **(Gallucci and Matzinger, 2001; Di Virgilio, 2005).** The danger signal or signal zero is followed by first signal. The first signal is generated by interaction of MHC-peptide complex at the surface of APC with the T cell receptor (TCR) on the T cell. The first signal confers specificity to the immune response. However, this primary signal is not sufficient to completely activate T cells. To activate T cell, a second, nonspecific co-stimulatory signal is often required. The co-stimulatory signal is induced by interaction of co-stimulatory molecules (CD80/86) on APCs with CD28 on T cells **(Lombardi et al.,**

2010). Co-stimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) belong to the B7 family of immune regulatory ligands. The lack of co-stimulation leads to anergy or apoptosis of antigen-stimulated T cells **(Orlikowsky et al., 2003)**. While presence of co-stimulation significantly lowers the activation threshold and allows naive T cells to be readily activated **(Lenschow et al., 1996; Greenwald et al., 2005)**. T cell activation is regulated in a well-organized manner. The interaction of CD80/86 on APC with CD28 on T cell activates the T cell while the interaction of CD80/86 with CTLA4 (Cytotoxic T-Lymphocyte Antigen 4) on T cell suppresses T cell activation. CTLA4 compete for CD80/86 ligation with CD28 to maintain the T cell activation **(Slavik et al., 1999; Berg and Zavazava, 2008)**. The CD28 is expressed on most resting and activated cells while CTLA-4 is generally restricted to activated T cells **(Rudd, 2009)**. Besides the negative signaling function of CTLA-4, CTLA-4 expression promotes the down-regulation of CD28 via enhanced internalization and degradation of CD28 **(Berg and Zavazava, 2008)**. DC dynamically regulate CD86 levels depending on their need to harness or reduce their T cell-activating ability. DC up regulate CD86 expression following their contact with microbial antigens **(Mellman and Steinman, 2001)** while DC down regulate CD86 expression following exposure to immune suppressive cytokines such as IL-10, produced either by other immune cells or by DC, itself **(De Smedt et al., 1997; Corinti, Albanesi et al., 2001; Haase et al., 2002)**. Although CD80 and CD86 are predominantly found on DC and professional antigen-presenting cells such as macrophage and activated B cells, they can be induced on other cell types including T cells **(Azuma et al., 1993; Inaba et al., 1994**). In general, CD86 is the more abundant in

terms of expression, and is up regulated more rapidly upon activation on APC. In contrast to CD86, CD80 is not generally found on resting APC and is induced more slowly upon cellular activation **(Sansom, 2000)**. The CD86 expression on APC is up regulated in presence of granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN gamma **(Freedman et al., 1991; Larsen et al., 1994; Liu et al., 1999),** IFN-alpha **(Radvanyi et al., 1999)**, IFN-beta **(Wiesemann et al., 2008)** and LPS (lipopolysaccharide) **(Ding et al., 1993).** There is also evidence suggesting that CD86 expression can be induced or enhanced on lymphocytes. IL-4 induces CD86 (B7.2) in B cell within 6 hr of treatment **(Stack et al., 1994)** similarly IL2 up regulated the CD86 expression on human CD4+ and CD8+ T cells **(Paine et al,. 2012).** Type I interferon, most likely IFN-beta, is crucial in the pathway leading to CD86 upregulation induced by both LPS and poly (I:C). Trif/ Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule (TICAM-1) acts as a common adapter molecule in TLR3- and TLR4-mediated responses that lead to upregulation of CD80 and CD86 while poly(I:C) up regulate the CD80/CD86 expression mediated in part by a TLR3-Trif-independent pathway **(Hoebe et al., 2003)**. The role of Membrane-Associated RING-CH Protein (MARCH1) in CD86 regulation has also been demonstrated **(Ohmura et al., 2009).** MARCH1 is a membrane-anchored E3 ubiquitin ligase that has previously been shown to ubiquitinate MHC class II molecule through lysosomal degradation **(Ohmura et al., 2006; Matsuki et al., 2007).** MARCH1 upregulation inversely correlated with CD86 expression **(Thibodeau et al., 2008).**

Viruses have evolved numerous strategies to avoid the host immune response via antigen presentation (Figure 1-2, Table 1-1)

Virus	Encoding Proteins	Mechanism	Effect	References
The Epstein–Barr virus (EBV)	EBV nuclear antigen 1 (EBNA1)	Escaping the processing by the proteosomes	Reduced MHCI presentation	**(Levitskaya et al., 1995)**
The Epstein–Barr virus (EBV)	BILF1	Degradation of MHC-I via lysosomes	Reduced MHCI presentation	**(Zho, 2011)**
Kaposi's sarcoma-associated herpes virus (KSHV)	Latency-associated nuclear antigen1 (LANA1)	Prevents viral protein degradation	Reduced MHCI presentation	**(Kwun et al., 2007)**
Herpes Simplex Virus (HSV)	Immediate early infected cell peptide 47 (ICP47) protein and Unique short 6 (US6) glycoprotein	Blocks the TAP mediated peptide transport	Reduced MHCI presentation	**(Fruh et al., 1995 ; Hill et al., 1995; Hengel et al., 1997)**
Human Cytomegalovirus (HCMV)	Immediate early infected cell peptide 47 (ICP47) protein and Unique short 6 (US6) glycoprotein	Blocks the TAP mediated peptide transport	Reduced MHCI presentation	**(Fruh et al., 1995 , Ahn et al., 1997; Hengel et al., 1997, Lehner et al., 1997)**
Human Cytomegalovirus (HCMV)	Unique short 3 (US3).	Inhibits tapasin activity	Reduced MHCI presentation	**(Lee et al., 2000)**
Bovine Herpes Viruses	Unique long 49.5 (UL49.5)	Degrades the TAP protein	Reduced MHCI presentation	**(Koppers-Lalic, et al.; 2005)**
Equine Herpes Viruses	Unique long 49.5 (UL49.5)	Blocks the binding of ATP to TAP	Reduced MHCI presentation	**(Koppers-Lalic, et al.; 2005)**
Cowpox virus encodes	CPXV203 protein	Help in retaining the MHCI molecules in the ER	Reduced MHCI presentation	**(Byun et al., 2007)**
Human immunodeficiency virus **(**HIV)	Nef protein	Down-regulates the surface expression of CD80 and CD86	Reduced T cell activation	**(Chaudhry et al., 2005; Shen et al., 2010)**

Table 1-1. Viral mechanisms to cause immunosuppression.

Only a few studies have investigated the effect of BVDV on MHC expression. The antigenically identical pair of non-cytopathic (Pep515ncp) and cytopathic (Pep515cp) BVDV isolates were used to determine their effect on MHCI and MHCII expression on MDDC. The ncp BVDV increased MHCI expression while it reduced MHCII and CD86 expression in MDDC. The cp BVDV reduced MHCI, MHCII and CD86 expression in MDDC in 48 hrs post infection **(Glew et al., 2003).** The Canadian 24515 field isolate of BVDV reduced the expression of MHCII and CD21-like **(**B-B7**)** on bovine peripheral blood mononuclear cells (PBMCs) **(Archambault et al., 2000)**. The bovine macrophages infected with cp and high virulence ncp strains of BVDV downregulated MHC II expression, while the high virulence strains had no effect on MHC II expression. In contrastcp strains of BVDV up regulated MHCI **(Chase et al., 2004)**. An analysis of mononuclear cells from lymph node and Peyer's Patch as revealed decreased expression of MHC II-expressing in from BVDV-infected calves at 9^{th} day p.i. **(Brodersen and Kelling, 1999)**.

1.6 Dendritic cells:

Dendritic cells (DC) are a subpopulation of morphologically distinct adherent mononuclear cells **(Mosier, 1967)**. DC are morphologically distinct cells isolated *in vitro* from peripheral lymphoid organs of mice and were initially discovered in mouse spleens. DC do not represent morphological variants of either lymphocytes or macrophages. In 1999, the first *in vitro* monocyte derived DC were generated from mice in 1999 **(Randolph et al., 1999)**. They lacked lymphocyte surface differentiation markers and do not exhibit the endocytic capacities of macrophages **(Steinman and Cohn, 1974)**.

DC can be distinguished from other members of the monocyte lineage (i.e. monocytes and macrophages) by their unique morphology. Immature DC are active phagocytic cells. Immature DC are scattered in close proximity to body surfaces, where they constantly sample the environment for antigen. As soon as any foreign antigen enters into body, immature DC engulf it. The uptake of antigen by immature DC can occur by several different mechanisms such as constitutive macropinocytosis **(Sallusto et al., 1995)** FcR-mediated endocytosis **(Sallusto and Lanzavecchia, 1994)**, caveaolae formation **(Werling et al., 1999)** and phagocytosis **(Reis e Sousa et al., 1993)**. The endosomes containing antigen fuse, with lysosomes and form an endolysosome. In endolysosomes, antigen is processed via proteolytic enzymes into peptides. These peptide antigens are presented in the groove of a major MHCII (Figure 1-2). The MHCII molecules are synthesized in ribosome. The MHCII molecules form a tricomplex containing alpha and beta chains and invariant Class II-associated invariant chain peptide (CLIP) peptide molecule (Figure 1-2). The invariant CLIP peptide molecule temporarily occupies the groove between alpha and beta chains. This tricomplex is transported to the lysosome through the Golgi apparatus. The foreign antigen is phagocytized and is contained in the endosome (Figure 1-2). The endosome fuses with the lysosome for antigen processing. Following the fusion, the CLIP peptide is removed from the grove. The free grove of MHC II molecule is occupied by the peptide antigenic fragment. The MHCII molecule containing antigen complex moves to the plasma membrane and presents it to T cell (Figure 1-2).

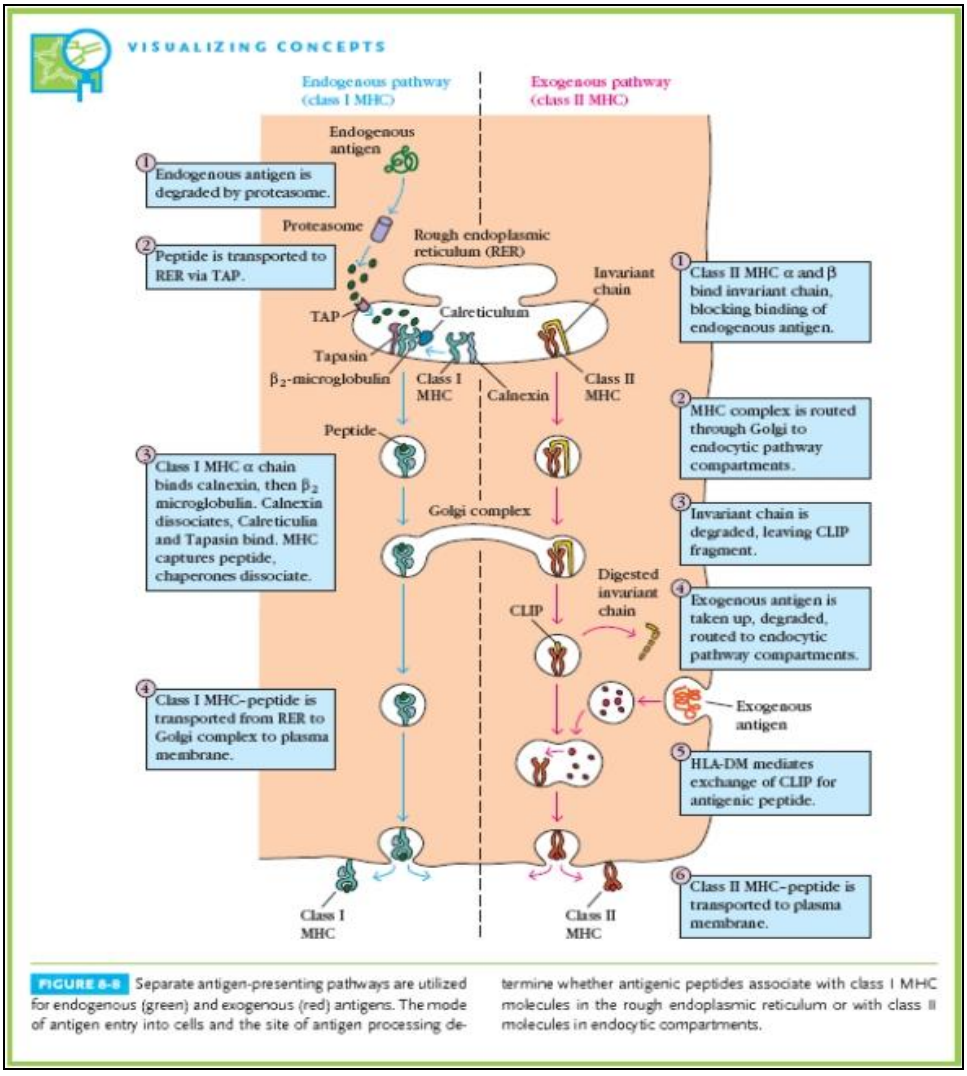

Figure 1-2 MHCI and MHCII antigen presentation. Owen et al., 2013 Kuby Immunology. W. H. Freeman Publishers Ltd, New York.

The formation of the central supramolecular activation complex (C-SMAC) results in the initial immunological synapse (the first activation signal). The C-SMAC comprises interaction of TCR with MHCII containing antigen peptide, MHC II with CD4 molecule, CD2 with CD2L, and CD28 (on T cell) with (CD80/86, B7) on DC. Interaction

between CD28 and CD80/86 provides co-stimulation that amplify signal from TCR. An additional interaction also occurs between the LFA-1 and ICAM-1 adhesion molecules in the peripheral supramolecular activation complex (P-SMAC). All of these interactions result in formation of the complete immunological synapse. The immunological synapse activates the T cell. The activated T cell produces IL-2 (Interleukin-2), IL-2 increases T cell proliferation and prevents the induction of anergy and cell death. In DC, PAMPs (pathogen-associated molecular patterns) are also recognized by pathogen recognition receptors (PRRs). The PRRs are present either on surface of DC or in its cellular compartments (Figure 1-3).

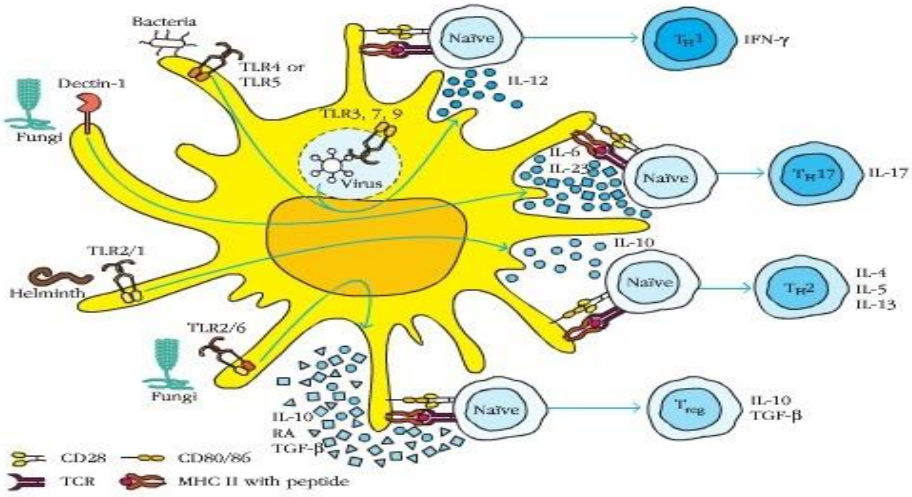

Figure 1-3 DC with Pathogen Recognition Receptors and T cell Activation Owen et al., 2013 Kuby Immunology. W. H. Freeman Publishers Ltd, New York

The PRRs recognize pathogen-associated molecular patterns (PAMP) like LPS **(Rescigno et al., 1999),** bacterial DNA **(Akbari et al., 1999)** and double-stranded RNA **(Cella et al., 1999).** Interaction of PAMPs and PRRs activates signal-transduction.

Signal-transduction results in expression of variety of immune-response genes. Immune-response genes are translated into various cytokines like TNF, IL-6, IL-12, IL-10, IL-17 and IFN-α that activate cells of innate as well as adaptive immune system. Recognition of PAMPs via PRRs along with stimulation by pro-inflammatory cytokines leads to activation and maturation of DC. During maturation, DC change from being a specialized phagocyte cell to professional antigen presenting cell. The mature DC home to secondary lymphoid organs to present antigen to T cells. The immature DC express CCR5 on their surface while activated DC decrease expression of CCR5 and up regulate chemokine receptor CCR7 **(Lei and Hostetter, 2007).** The DC also produce anti-microbial proteins and peptides such as defensins and members of the complement system.

1.7 DC subsets in other species:

DC can be generated from a number of different precursor cells with *in vitro* using cytokine-supported culture. They can be derived from purified, primitive self-renewing haemopoietic stem cells as well as lineage-restricted common lymphoid progenitors (CLP), common myeloid progenitors (CMP), granulocyte/macrophage-restricted precursors and pro-T cells **(Manz et al., 2001).** DC can also be generated from populations of Lin^- (lineage negative) CD117 $(c\text{-}kit)^+$ BM or fetal liver cells **(Zhang et al., 1998)** or from 'CD4 low' T precursor cells in the thymus **(Ardavinet al., 1993)** or from peripheral blood mononuclear cell **(Schreurs et al., 1999)**. Mice have two distinct DC subsets, myeloid DC and lymphoid DC. $CD11c^+MHCII^-B220^+CD19^-$ blood DC precursor can develop from either CMP or CLP. The CMP generates myeloid DC while the CLM develops into lymphoid DC in mice. Both the subsets express high levels of

CD11c, MHCII and CD86 and CD40. The myeloid and lymphoid DC can be distinguished on the basis of CD8a, CD1d and DEC 205 expression. CD8a is expressed on the lymphoid DC while it is absent in myeloid subset. The DEC-205 and CD1d are expressed with higher levels on lymphoid DC but they can be up regulated on myeloid DC by *in vitro* culture **(Wu et al., 1996; Vremec and Shortman 1997)**. The lymphoid DC make higher levels of interleukin (IL)-12 that induces production of IFN gamma. The lymphoid DC have less phagocytic capacity than myeloid DC **(Pulendran et al. 1997; Leenen et al. 1998)**. The Flt3 ligand (Flt3-L) and GM-CSF can expand mature DC in mice. Both lymphoid and myeloid DC increase in number following Flt3-L injection **(Maraskovsky et al., 1996)**. The Flt3-L treatment leads to an increase in DC numbers in multiple organs in mice, including spleen, lymph nodes, blood, thymus, Peyer's patch, liver and lungs. In contrast, GM-CSF preferentially expands the myeloid DC subset *in vivo* **(Pulendran et al., 1999).**

In humans, three subsets of DC have been identified. Two subsets originate from CD34+ myeloid precursors while the third subset originates from CD34+ lymphoid precursors (Figure 1-4).

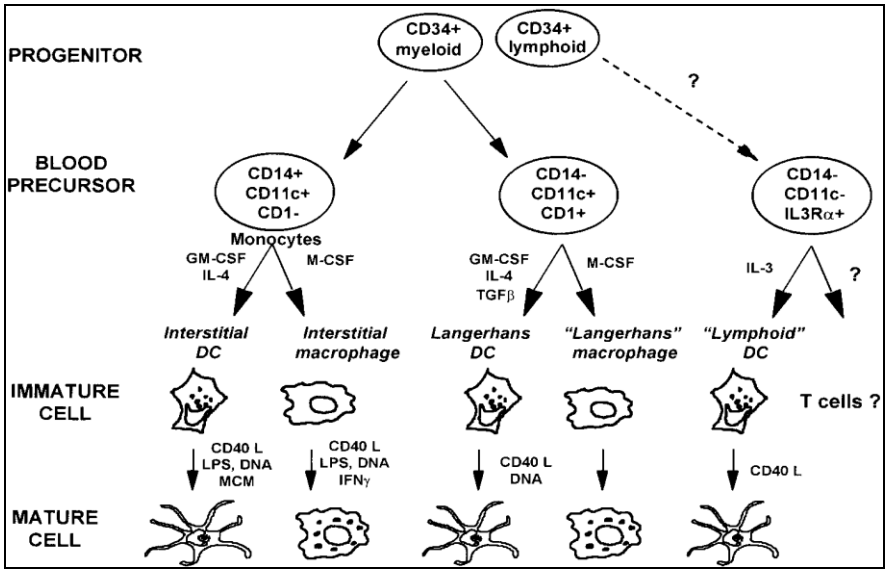

Figure 1-4 Human dendritic cell subsets. From Banchereau et al., 2000. Immunobiology of dendritic cells, *Annual Review Of Immunology*. 18:767-811.

The CD34+ lymphoid precursors give rise to plasmacytoid and Langerhans dendritic cells. The plasmacytoid DC produce high amounts of IFN-alpha **(Banchereauet al., 2000)**. The CD34+ myeloid progenitors differentiate into monocytes (CD14+ CD11c+ DC precursors) that are further differentiated into immature DC in response to granulocyte/macrophage colony-stimulating factor–positive (GM-CSF) and interleukin-4 (IL-4) or macrophages in response to macrophage colony stimulating factor (M-CSF). The myeloid progenitors can also differentiate into CD11c+ CD14- precursors, which yield Langerhans cells in response to GM-CSF, IL-4 and transforming growth factor (TGF) beta **(Banchereau et al., 2000)**. The $CD14^{-}$ $CD11c^{+}CD1a^{+}$ Langerhans cell precursors migrate into the skin epidermis and become Langerhans cells while the CD14+ $CD11c^{+}CD1a^{-}$ cells migrate into the skin dermis and other tissues to become

interstitial DC **(Liu, 2001)**. The myeloid DC express all TLR except TLR7 and TLR9 while the plasmacytoid DC selectively express TLR7 and TLR 9.

1.8 Monocyte-derived dendritic cells:

DC are continuously produced in the bone marrow from hematopoietic stem cells. After exiting the bone marrow, DC migrates into lymphoid and non-lymphoid tissues as epidermal Langerhans cells, splenic marginal zone dendritic cells and/or interstitial dendritic cells **(Banchereau and Steinman, 1998).** It is very difficult to isolate DC from these peripheral tissue sites without affecting their structure, viability and phenotype. Most experimental and clinical studies rely on the *in vitro* development of DC. The *In vitro* DC can be generated either from CD34+ progenitor cells or from non-proliferating blood monocytes **(Sallusto and Lanzavecchia, 1994)**. The monocyte is the most common source for *in vitro* generation of DC. Monocytes develop in the bone marrow and enter and circulate in the blood stream until they are recruited to extravascular compartments. The recruitment of monocytes occurs in inflammation as well as under steady-state condition to maintain the homeostasis of the monocyte cell system **(Leon and Ardavin, 2008)**. The monocyte-derived DC (MDDC) are generated by culturing monocytes with granulocyte-macrophage colony stimulating factor (GM-CSF) in the presence of either toll-like receptor ligand (TLR-L) or thymic stromal lymphopoietin (TSLP) **(Encabo et al., 2004)** or IL-4 **(Yi and Kwak, 2005)** or TNF alpha **(Tosi et al., 2004).** The monocytes may, particularly in the setting of inflammation, become MDDC that have DC-like phenotype (CD11c+MHCIIhi) and/or DC-like functions (ex. ability to stimulate naive T cells or cross present antigens to T cells) **(Geissmann et al., 2003;**

Fahlen-Yrlid et al., 2009). The differentiation of monocytes to DC may be affected by inflammatory mediators in the local environment **(Serbina et al., 2008).**

1.9 Bovine Monocyte-derived DC (MDDC):

The most common procedure to generate DC is culturing blood monocytes in the presence of GM-CSF and IL-4 **(Romani et al., 1994).** The bovine MDDC express moderate to high levels of the co-stimulatory molecules (CD80/86), DEC 205 and MHC class II molecules. MDDC have a down regulated expression of the myeloid cell differentiation molecule CD14 as compare to monocytes and macrophages**(Beekhuizen et al. 1991; Bajer,Mahke et al., 2000; Garcia-Tapia et al., 2003;Flores-Mendoza et al., 2012).**

1.10 Autophagy an important component of antigen presentation and innate immunity:

Autophagy is a cellular process where cytoplasmic components are sequestered and recycled to maintain cellular homeostasis **(Mizushima, 2007).** It is a normal physiological mechanism for the degradation of proteins and organelles. Regulation of autophagy is necessary to maintain metabolic equilibrium and immune homeostasis. Reduced autophagy is associated with cancer, neurodegeneration, and infectious diseases **(Heath and Xavier, 2009)**.

Autophagy acts as an innate immunity effector against intracellular bacteria and viruses **(Gutierrez et al., 2004; Nakagawa et al., 2004)** including herpes simplex virus-1 **(Orvedahlet al.,2007)** and vesicular stomatitis virus **(Shelly et al., 2009)**. Autophagy can be induced by interferon-gamma (**Inbal et al., 2002; Gutierrez et al., 2004, Dengjel et al., 2005; Paludan et al., 2005)**. The autophagy is induced by various toll-like receptors

(TLR), nucleotide-binding oligomerization domain (NOD)-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors, damage associated molecular patterns such as high-mobility group box 1 (HMGB1) protein **(Deretic, 2011).** The autophagy is induced by LPS through a Toll-interleukin-1 receptor domain containing adaptor inducing interferon beta (TRIF) dependent, myeloid differentiation factor 88 (MyD88) independent TLR4 signaling pathway**(Xu et al., 2007).**

The discovery of autophagy genes and its signaling pathways allowed the identification of the machinery involved in autophagosome formation, fusion, and degradation **(Klionsky and Emr, 2000; Huang and Klionsky, 2002).**. The positive-stranded RNA viruses such as poliovirus and coxsackie virus (**Jackson et al., 2005; Wong et al., 2008)**, the viruses from Flaviviridae family like hepatitis C virus (HCV) **(Dreux and Chisari, 2009)**, and dengue virus (DENV) exploit autophagy for their efficient replication **(Lee et al., 2008; Panyasrivanit, Khakpoor et al., 2009; Heaton et al., 2010).** The NS5A and NS5B proteins of HCV co-localize with autophagosomes with increased viral replication indicating that HCV replicates in autophagosomes and up regulates of autophagy to facilitate HCV replication (**Sir et al., 2012)**

There has been little work done to examine the role of BVDV with autophagy. An interesting finding was that a cp BVDV isolated from an animal died due to mucosal disease contained an insertion of cellular mRNA coding for light chain 3 (LC3), an autophagy marker **(Kuma et al., 2007).**. This finding indicated a possible role for BVDV replication with the autophagy machinery **(Fricke et al., 2004).**

SUMMARY

BVDV infection is associated with immunosuppression and increases the risk of secondary infections. An antigen-specific immune response is mounted by activation of the well-controlled T cell response via APC. APC process and present antigen to T cell through MHC molecules. The interaction of MHC-peptide complex with T cell receptor along with co-stimulation confers specific immune response. The co-stimulatory signal is induced by interaction of co-stimulatory molecules (CD80/86) on APC with CD28 on T cell. Lack of co-stimulation leads to anergy or apoptosis of reactive T cells. BVDV infects a wide variety of cell types and has a predilection for cells of the immune system including antigen-presenting cells like monocytes that develop into DC. DC are the most important APC that activate naïve T cells. It is hypothesized that the virus infection of DC may interfere with surface marker expression, antigen presentation and T-cell activation. Interference with DC activity may be one of the causes of BVDV immunosuppression. The infected DC may also potentially play an important role in dissemination of the virus . it is difficult to study DC *in situ* is difficult without affecting their viability and phenotypic characteristics. As an *in vitro* DC model, DC can be differentiated from their monocyte precursor into monocyte-derived dendritic cells (MDDC). In the current study, a reproducible method to differentiate large number of MDDC was optimized. MDDC were used as *in vitro* model to DC to investigate effect of BVDV with different virulence levels and biotypes, on MHCI, MHCII and CD86 expression. The ability of BVDV-infected MDDC to maintain and produce infectious

BVDV was studied to determine a possible role for DC in dissemination of BVDV infection.

In the current study, the role of BVDV on autophagy induction and use of autophagy machinery for its effective replication was investigated. Finally this study also studied the primary *in vivo* humoral antibody response against cytopathic or noncytopathic BVDV infection was investigated.

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CHAPTER 2.

RESTRICTED REPLICATION OF BVDV IN BOVINE MONOCYTE-DERIVED DENDRITIC CELLS

ABSTRACT

Bovine viral diarrhea virus (BVDV) is one of the most economically important diseases of ruminants. Dendritic cells (DC) are important antigen presenting cells and are present in skin or mucosal surfaces for active surveillance of the antigens. The immature DC are highly phagocytic cells that capture, process and present antigen to T cell in secondary lymphoid organs. Tissue-resident DC that migrate from peripheral sites to lymphoid organs are essential in the initiation of adaptive immune response and for the maintenance of peripheral tolerance. The BVDV-infected DC may play an important role in BVDV dissemination in the body. In this chapter, bovine monocytes were cultured with bovine recombinant GM-CSF (100ng/ml) and IL-4 (200ng/ml). Over a period of 5-7 days in culture, the monocytes differentiated into monocyte-derived dendritic cells (MDDC). The MDDC had morphological and phonotypical characteristics similar to classical dendritic cells. The MDDC were positive for MHCI, MHCII, DEC205 and CD86 and negative for CD21 and CD14. During differentiation of MDDC, it was found that monocytes from Brown Swiss calves had higher capability to differentiate MDDC than the monocytes from Holstein Friesian calves. It was found that 20-30% isolated monocytes from Brown Swiss calves e differentiated in MDDC while only 5-10% monocytes isolated from Holstein Friesian differentiated in to MDDC. Further, the ability of BVDV to replicate in bovine monocyte-derived dendritic cells (MDDC) or its

progenitors (monocytes or intermediate MDDC) was investigated. MDDC were differentiated from blood monocytes by *in vitro* culturing. Over a period of 5-7 days in culture, the monocytes differentiated into MDDC. The culture, which depending on time point was made up of fully differentiated MDDC, monocytes or intermediate stages of MDDC were infected with BVDV at 2, 3, 4 or 5 days of culture were infected with BVDV. Four strains of BVDV were used in this study: the high virulent BVDV2a- 1373, typical virulent BVDV2a-28508-5, and a virus pair, cytopathic (cp) BVDV1b-TGAC and noncytopathic (ncp) BVDV1b-TGAN recovered from an animal that died of mucosal disease. The cells were infected at a multiplicity of infection (MOI) of 6 with each of the viruses. The supernatant and cells were harvested at regular intervals for up to 192 hr p.i. The Virus titer was determined by virus isolation and viral RNA using qRTPCR in cell lysate at each time point. The results showed no virus in MDDC and MDDC supernatant following BVDV infection while viral RNA increased in MDDC through 144 hr after infection. The kinetics of viral RNA production along with the amount of viral RNA was significantly different between the different viral strains. The lowest amount of cell associated viral RNA was observed in MDDC infected with the ncpBVDV2a-28508-5 strain of BVDV while maximum viral RNA accumulated occurred in the MDCC infected with the high virulent BVDV2a-1373 strain or cp BVDV1b-TGAC strain of BVDV. Virus replication was then compared between MDDC and monocytes. The monocytes supported the production of infectious virus. The peak virus titer for all 4 strains in monocytes was observed at 72 hr p.i. although the amount of virus varied by strain. The intermediate stage of MDDC produced viral RNA up to 4 days (96 hr) after infection.

The titer of infectious virus decreased with MDDC differentiation and completely stopped at 5 days (120 hr) of differentiation. These findings suggested the ability of MDDC to produce infectious virus reduced with MDDC differentiation and was completely lost in fully developed MDDC.

INTRODUCTION

Since the first discovery of BVDV in the 1940s, it has been an important disease in ruminants around the world. The economic importance of BVDV is increasing with the emergence of seemingly more virulent viruses, as evidenced by outbreaks of hemorrhagic syndrome and severe acute bovine viral diarrhea beginning in the 1980s and 1990s **(Goens, 2002)**. The genetic diversity between different strains of BVDV classified the BVDV in two genotypes on the basis of highly conserved 5'UTR region. These two groups are BVDV Type I and BVDV Type II **(Ridpath, 2003).** The survey report indicated that three subgenotypes, BVDV-1a, BVDV-1b, and BVDV-2a are commonly circulating in the United States. **(Ridpath et al., 2011).** BVDV is capable of a wide spectrum of disease and clinical symptoms. The severity of the disease can range from mild acute infection to severe infection depending up on both on the virulence of the strain and the state of the infected host. BVDV infection can be manifested clinically as acute infection, severe acute infection, chronic infection, congenital infection, persistent infection or mucosal disease **(Pritchard, 1963; Brownlie, 1990; Moennig and Liess, 1995; Peterhans et al., 2010).** BVDV is an important immunosuppressant viral disease. The level of suppression of the adaptive immune response is strain dependent **(Chase, 2013)**. The ncp BVDV evades the immune system of pregnant dam and establishes a

persistent infection (PI) in the fetus during 40-120 days of gestation **(Chase et al., 2004)**. PI calves are immunotolerant and remain a source of infection to other animals. Superinfection of PI animals with antigenically homologous cp BVDV strain results in fatal mucosal disease **(Brownlie et al., 1984)**.

Dendritic cells (DC) are important antigen presenting cells (APC) that play a crucial role in mounting the immune response. DC provide active surveillance to monitor invading pathogens and present it to the immune system. After engulfing the pathogen, the DC migrate from peripheral sites to lymphoid organs to initiate the adaptive immune response. The infected DC may play an important role in the dissemination of BVDV in the body and facilitate in the establishment of infection. DC are susceptible to various pathogens including BVDV. BVDV infected DC may have altered cell surface marker expression that are necessary to mount the effective immune response. Altered cell surface marker may facilitate the establishment of persistent infection. DC infection with cp strain of BVDV does not show CPE (cytopathic effect). The presence of BVDV viral protein in DC indicated that BVDV replicate in MDDC **(Glew et al., 2003)**.

In this study, we optimized a simple, reproducible culture system that allows monocytes to mature and differentiate into MDDC with high yield and viability. The cells generated were confirmed as DC by morphological and phenotypical characteristics and investigated the ability of BVDV to replicate in the various stages of MDDC from monocyte to fully differentiated MDDC. Four strains of BVDV were used in this study including the high virulent BVDV2a-1373, typical virulent BVDV2a-28508-5, and a

virus pair cytopathic (cp) BVDV1b-TGAC and noncytopathic (ncp) BVDV1b-TGAN recovered from an animal that died of mucosal disease.

MATERIALS AND METHODS

Animals

Eighteen (18) Holstein Friesian and six (6) Brown Swiss female calves (8-12 months of age) housed at Dairy Farm, South Dakota State University (SDSU), Brookings, SD, USA were used in this study. All animals were healthy. The SDSU Institutional Animal Care and Use Committee approved animal handling and blood collection.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated as per method previously described **(Ulmer et al., 1984)** and differentiated into MDDC **(Mwangi et al., 2005)** with following modifications; 2-mercaptoethanol, 2 mM GlutaMax and 25 mM HEPES were replaced with 1mM sodium pyruvate (personal communication, Dr Waithaka Mwangi, Texas A&M University, USA) and the medium used for MDDC differentiation was RPMI-1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100µg/ml), bovine recombinant granulocyte macrophage-colony stimulating factor (GM-CSF (100ng/ml) and IL-4 (200ng/ml). In the previous method fresh, complete RPMI-1640 medium and cytokines was added at every 3rd day while in current study 750 µl fresh, complete RPMI-1640 medium with cytokines was added every 2nd day (every other day). The addition of sodium pyruvate and increasing the concentration of FBS from 10% to 20% improved the

differentiation of MDDC. The beneficial effect of sodium pyruvate may be due to its protective effect to nutrients in media and providing energy to the cell **(Giandomenico et al., 1997).**

Briefly, sixty (60) ml of heparinized venous blood was collected from healthy calves. The buffy coat layers were separated by centrifuging the blood at 1100g for 30 minutes at 4°C. The cells from buffy coat layer were suspended into heparinized (10 U/ml) PBS in 1:3 ratio. The diluted cells were overlaid on 3ml, 65% Percoll (GE Healthcare Biosciences**,** Pittsburgh, PA, USA) in 15 ml conical tubes (Falcon, Oxnard, CA, USA) and centrifuged for 30 min, at 1100g at 4°C. The white cell layer of PBMC was aspirated by pipette from the interphase. The collected PBMC were suspended in heparinized PBS (10 U/ml) and pelleted by centrifugation at 1700 rpm for 15 min at 4°C. The PBMC were washed two times by suspending them in PBS and centrifugation at 1700 rpm for 15 min at 4°C. Finally, PBMC were suspended in RPMI 1640 medium supplemented with 10% FBS, 1mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100µg/ml) to achieve final concentration of 1x 10^7cells/ ml. Three (3) ml of the cell suspension was added to each well of a 6-well plate (Falcon, Oxnard, CA, USA) and incubated at 37°C in a humidified CO_2 incubator for 3 hr.

Separation of adherent monocytes

The monocytes were isolated by the plastic adhesion method **(Mwangi et al., 2005)**. The PBMC were cultured in 6-well plates for 3 hr at 37°C in a humidified CO_2 incubator. The unattached cells were discarded and plates were washed 4 times with PBS. The attached cells were detached with Accuatase (eBioscience, San Diego, CA, USA).

Detached cells were washed two times by suspending them in PBS and centrifugation at 500g for 15 min at 4°C.

Monocyte-derived dendritic cell (MDDC) culture

The accuatase-detached cells were characterized as $MHCI^+$ $MHCII^+$ and $CD14^+$ monocytes based on flow cytometer analysis done on a FACScan (Becton-Dickson, Mountain View, CA) using MHC-I (H58A), MHC-II (H42A), CD21 (BAQ15A) andCD14 (MM61A) primary antibodies (VMRD Inc., Pullman, WA, USA). The harvested CD14+ monocytes were counted and the yield determined. The monocytes were differentiated into monocyte-derived dendritic cells (MDDC) **(Mwangi et al., 2005)** with some modifications as described above. Briefly, the monocytes were diluted in RPMI 1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 µg/ml), bovine recombinant GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) kindly provided by Dr. Waithaka Mwangi (Texas A&M University, USA) to achieve the final concentration of $5x10^5$cells/ml. Three (3) ml of this cell suspension was added to each well in 6-well plates. The cells were incubated at 37°C in a humidified CO_2 incubator for 7 days. Seven hundred and fifty µl (750 µl) of RPMI 1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100µg/ml), bovine recombinant GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) was added to each well at alternate day. At day seven, differentiated cells were collected and examined for morphological and phenotypical characteristics. The MDDC were either loosely attached or floating. The viability of loosely attached or floating MDDC was determined by trypan blue exclusion assayThe trypan blue exclusion assay

was done to determine the cell viability by staining the MDDC with 0.4% trypan blue stain **(Strober , 2001).** Briefly, the 500 µl MDDC aliquots of cell suspensions from 6-well plates was centrifuged at 200g for 2 minutes in 1.5 ml tubes and cell pellet was suspended in 200 µl PBS. Two hundred (200) µl of 0.4% trypan blue was added to the cell suspension. The cells were incubated for 3-4 minutes at room temperature and examined under microscope. A total 100 cells were examined. The non-stained viable cells were counted and cell viability was calculated using the following formula:

Cell viability percentage = Number of viable cells (none trypan blue stained cells) cells X 100/Total counted cells.

Characterization of MDDC

Morphological characterization

The cells were examined at day 1, 4 and 7 days of incubation using an inverted compound microscope at 40x (Olympus, PA, USA) to determine morphological changes. Cells were examined for changes in size and shape and dendrites formation. Photomicrographs were taken at each time point. The percentage differentiation of monocytes to MDDC was determined at day 7 of culture. During differentiation, cells were also considered for their animal source (Brown Swiss and Holstein Friesian) to determine the effect of breed on MDDC differentiation.

Phenotypic characterization using flow cytometry.

For flow cytometry analysis, monocytes or the differentiated MDDC were collected by removing the media containing the cells from the 6-well plates by gentle mixing and aspiration. After collection, the cell suspension was centrifuged for 10 min at

200g and washed 1x with PBS. Cell number was adjusted to $1x10^6$/ml. Six primary mouse mAb antibodies for MHC-I (H58A), MHC-II (H42A), CD86 (IL-A190A), CD21 (BAQ15A) andCD14 (MM61A)(VMRD Inc., Pullman, WA, USA) and DEC205 (Kindly provided by Dr. Waithaka Mwangi, Texas A&M University, USA) were used to characterize the cells. The primary antibodies were diluted 1:100 in PBS containing 1% FBS. The 100 µl of cells suspension was incubated with 50µl of diluted primary antibodies at 4°C for 10 min followed by washing by centrifugation at 200g for 4 minutes at 4 °C in round bottom 96 well plates. After centrifugation cell pallet was suspending them in 200 µl PBS. After primary staining and washing, cells were incubated with 50 µl FITC labeled anti-mouse secondary antibody (VMRD Inc., Pullman, WA, USA) which was diluted 1:1000 dilution in PBS containing 1% FBS at 4°C for 10 min. After incubation with secondary antibody, cells were washed two times with PBS. Cells were suspended for in 200 µl of 1% paraformaldehyde for fixing. The cells fixed in1% paraformaldehyde analyzed using FACScan (Becton-Dickson, Mountain View, CA). At least 20,000 cells were run at each time points and each experiment was repeated at least 3 times.

Virus:

The four BVDV strains were used in this study included a pair of cp/ncp BVDV1b viruses, Tifton GeorgiA Cytopathic (BVDV1b-TGAC) and Tifton GeorgiA Non-cytopathic (BVDV1b-TGAN) and recovered from an animal that died of mucosal Disease **(Brownlie et al., 1984; Fritzemeier et al., 1995; Ridpath et al., 1991)**, A high virulence noncytopathic strain from the BVDV2 species (BVDV2a-1373) isolated

following an outbreak of peracute BVDV **(Carman et al., 1998; Stoffregen et al., 2000)** and the typical virulence noncytopathic virus from the BVDV2 species isolated from an asymptomatic persistently infected calf (BVDV2a-28508-5) **(Liebler-Tenorio et al., 2003)** (Table 2-1).

Five (5) ml of $5x10^5$ MDBK cells/ml were seeded in T25 flasks and culture were grown to 60-70% confluency at the time of inoculation with virus. At the time of inoculation, the media was removed and 0.75 ml of virus inoculum with a multiplicity of infection [MOI] of one was added to each T25 flask. Virus was adsorbed for 1 hr at 37°C in a humidified CO2 incubator. After one (1) hr incubation, inoculum was removed and the cells were washed with sterile PBS. After washing, 5 ml of media was added to each flask. The cells were incubated at 37°C in a humidified CO2 incubator for 4-5 days for cultures infected with noncytopathic virus or or 70-80% cytopathic effect forcultures infected with cytopathic virus, TGAC. Cultures were harvested by two freeze thaw cycles. After 4-5 days for cultures infected with noncytopathic virus orof incubation cells were freezed at (-80˚C for 15 minutes followed by thawing at 25˚C).. The cell debris was pelleted by centrifugation at 1200g for 10 min at 4°C. The virus in the resulting supernatants was titrated and the supernatants were, aliquoted and stored at -80 °C for further use.

The virus titration was determined by Reed and Munech method **(Reed and Munech, 1938**). Briefly MDBK cells were detached from tissue culture flask. The number of cells was adjusted to $5x10^5$ cells/ ml. One hundred eighty (180) µl cell suspensions was added to each well of 96-well plate. Twenty (20) µl of virus was added to the first row of the

plate. The virus was mixed with MDBK cells and 20 µl of this dilution was added to next row to achieve 10 fold dilutions. No virus was added to the last two rows of wells. These wells served as negative controls. The four replicates of each dilution was performed to determine the virus titer. The plate was incubated at 37 °C at humidified incubator for next 4 days. The plate was examined every day for cytopathic effect (CPE) of the virus. The highest dilution showing CPE is used as end point to calculate the proportionate distance (PD). The PD was used to determine the viral concentration (TCID50) as per formula as described earlier (**Reed and Munech, 1938**).

1. Proportionate distance (PD) = (% CPE at dilution above 50%) – (50%)/ (% CPE at dilution above 50%)- (% CPE at dilution below 50%) (e.g. 60-50/60-0= 0.166)

2. Calculation of end point just next to 50% CPE and conversion into – Log (e.g.10^{-6} dilution would be -6)

3. Calculation of $TCID_{50.}$

4. $TCID_{50}$ for 20 µl= $^{(PD+ \text{ - Log dilution above } 50\%)}$ (e.g. $1x10^{6.166}$)

For ncp BVDV, the same procedures with the exception of determining the end point since no CPE were produced. The end point for ncp BVDV was determined by staining the MDBK cells with anti-BVDV antibody (IDEXX Laboratories, Westbrook, ME, USA) followed by biotinylated rabbit anti-mouse IgG (Zymed, Invitrogen Corporation, Frederick, MD, USA) Steptavidin-HRP (Invitrogen Corporation, Camarillo, CA, USA) and AEC reagent (3 amino-9 ethyl-carbazole) (Sigma-Aldrich, St. Louis, MO, USA). The

end point for ncp BVDV was determined by the presence of red stained cell showing BVDV protein.

The Cooper strain of bovine herpesvirus 1 (BHV-1) was used as a positive control as it had been previously shown to infect and produce infectious virus in monocyte-derived macrophages (MDM**) (Elmowalid, 2003).**

Madin Darby bovine kidney (MDBK) cells:

BVDV-free MDBK cells (passage 95-110) were grown in MEM (pH 7-7.4) supplemented with 10% BVDV free fetal calf serum (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 μg /ml). MDBK cells were used for viral propagation and titration of BVDV and BHV-1.

MDDC, MDBK cells or Monocyte infection:

The fully differentiated MDDC were collected at 7 days of culture. The MDDC were centrifuged at 1700 rpm at 4°C for 15 min in 15 ml conical tubes and suspended in RPMI 1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U /ml) and streptomycin (100 μg /ml), bovine recombinant GMCSF (100 ng/ml) and IL-4 (200 ng/ml) to achieve final concentration $5x10^5$/ml. One (1) ml of cell suspension was added to each well of 24-well plates and infected with virus at 6 MOI with one of BVDV strains. Because the cells were in suspension, the inoculum was not removed from the MDDC. Mock-infected MDDC were used as controls. The BVDV infected MDDC and their supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 72 hr, 96 hr, 120 hr, 144 hr, 168 hr or192 hr p.i. The MDBK cells produce infectious BVDV virus. Because of the permissive nature of MDBK cells for BVDV, the MDBK cells were used

as a positive control for BVDV growth at each time point. The MDBK cells were detached from tissue culture flask by 0.25% trypsin. The MDBK cells were adjusted at cells $5x10^5$ cells /ml. One (1) ml of cell suspension was added to each well of 24-well plates and MDBK cells were attached overnight. MDBK cells were infected with virus at 6 MOI of infection with each one of the BVDV strains. Virus was adsorbed for 1 hr and non-adsorbed virus was removed by washing with PBS. MDBK cells were supplemented with MEM (pH 7-7.4) supplemented with 10% BVDV free fetal calf serum (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 μg /ml). The BVDV infected MDBK cells and their supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 72 hr, 96hr, 120 hr, 144 hr, 168 hr or192 hr p.i as was done for MDDC.

For monocyte infection, freshly collected monocytes were adjusted to 5 $x10^5$ cells /ml in RPMI 1640 medium supplemented with 10% FBS, 1mM sodium pyruvate, penicillin (100 U /ml) and streptomycin (100 μg /ml). Monocytes were infected with one of the virus of BVDV at MOI of 6. Virus was adsorbed for 1 hr. The non adsorbed virus was removed by washing the monocytes with PBS. The monocytes were washed by suspending them in PBS and centrifugation at 1700 rpm for 15 min at 4°C. Finally monocytes were suspended in RPMI 1640 medium supplemented with 10% FBS, 1mM sodium pyruvate, penicillin (100 U /ml) and streptomycin (100 μg /ml) to achieve final concentration $5x10^5$ cell/ml. One ml of this cell suspension was added to each well of 24 well plates. The BVDV infected monocytes and their supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 72 hr, 96 hr, 120 hr, 144 hr, 168 hr or192 hr p.i.

The BVDV titer in MDDC, monocytes or in MBBK cells and their supernatant was measured at each time points as per the method described above (**Reed and Munech, 1938**). The MDM (monocyte-derived macrophage) has been shown to be susceptible to BHV1 infection and produce infectious virus **(Elmowalid, 2003)**. Because of the similar nature of MDM and MDDC, the Cooper strain of BHV1 was used as a positive virus control for MDDC infection. MDDC or MDBK cells were infected with Cooper strain of BHV1 with 6 MOI of infection. The BHV1 infected MDDC or MDBK cells and their supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 72 hr, 96 hr p.i. and titrated for virus production as per method described earlier (**Reed and Munech, 1938**).

For infectious BVDV production studies in the intermediate stage of MDDC, MDDC were collected at 2 (48 hr), 3 (72 hr), 4 (96 hr) or 5 (120 hr) days of differentiation. The cells were infected with the 1373 strain of BVDV at 6 MOI of infection. The cells of intermediate stage of MDDC were collected at 24 hr or 48 hr p.i. for virus titration per method described earlier (**Reed and Munech, 1938**).

UV inactivation of Virus:

The current study revealed that BVDV infect and replicate viral RNA in MDDC but do not produce infectious virus. To validate the result for viral RNA replication, the UV inactivated 1373 strain of BVDV was used as negative control. The virus was UV inactivated as per method described earlier (**Fortunato et al., 2000)** with some modifications. Briefly, three (3) ml of 1373 (virus titer 7 log10/ml) strain of BVDV was placed in 5 ml glass beaker. The virus was exposed to 302 nm wavelength UV irradiation (Ultra-Violet Products Inc, San Gabriel, CA, USA) for 15 minutes at a

distance of 2 mm. The sodium pyruvate (5 mM final concentration) was added to the inoculum immediately after irradiation. The inactivation of viruses was confirmed by immunohistochemistry (IHC) as described below. The MDBK cells were treated with UV-irradiated virus with equivalence to MOI of 6. The live high virulent BVDV2a-1373 strain with same MOI was used as positive control. One (1) ml MDBK cell suspension ($5x10^5$ cells/ml) was added to each well (4 wells) in 24-well plate. The MDBK cells were attached overnight at 37°C in a humidified CO_2 incubator. The MDBK cells were exposed to UV-treated virus or the live high virulent BVDV2a-1373 virus. The UV-treated or live virus was adsorbed for 1 hr at 37°C in a humidified CO_2 incubator. After the 1 hr incubation, the inoculum was removed and cells were washed with sterile PBS. After the washing, 1 ml MEM medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100μg/ml) was added to each well. The cells were incubated at 37°C in a humidified CO_2 incubator for 4 days. After 4 days, cells were fixed with 20% acetone. The fixed cells were stained for presence of BVDV protein using anti Erns BVDV antibody (Mab 15C5) (IDEXX Laboratories, Westbrook, ME, USA) followed by biotinylated rabbit anti mouse IgG (Zymed, Invitrogen Corporation, Frederick, MD, USA) Steptavidin-HRP (Invitrogen Corporation, Camarillo, CA, USA) and AEC reagent (3 amino-9 ethyl-carbazole) (Sigma-Aldrich, St. Louis, MO, USA) **(Baszler, Evermann et al. 1995)**. AEC produced red color in MDBK cells infected with live 1373 strain of BVDV but not in UV-treated virus, indicating UV irradiation inactivated the BVDV.

Viral RNA isolation and quantification through qRT-PCR in BVDV-infected MDDC, monocytes or in MDBK cells:

The differentiated MDDC, monocytes or MDBK cells were infected at a MOI of 6 with one of the four BVDV viruses. The cells were collected at 1 hr, 6 hr, 12 hr, 24 hr, 72 hr, 96 hr, 120 hr, 144 hr, 168 hr or 192 hr p.i. The cells were washed 3 times by centrifugation at 200g and suspending in sterilize PBS in 1.5 ml tubes at room temperature (25 °C). The cells were finally suspended in RNAase free water. The cells were lysed by freezing at -80 °C for 15 minutes and thawing at 4°C for 30 minutes. The cell lysate was centrifuged at 200g rpm at 25°C for 10 minutes. The supernatant of cell lysate was used to isolate the viral RNA using QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA, USA).

The quantification of viral RNA was done using qRTPCR (Stratagene MX3000P Real-Time Thermocycler (StratageneInc, La Jolla, USA) in 25µl reaction with FAM dye. The qRTPCR targeted the 5' untranslated region of viral genome (5'UTR) using following primers (forward: 5-GGGNAGTCGTCARTGGTTCG-3; BVDV reverse: 5-TGCCATGTACAGCAGAGWTTTT-3). These primers amplify a fragment of approximately 190 bases in both BVDV type I and BVDV type II **(Mahlum et al., 2002).** To compare the amount of viral RNA in samples at different time points, the standard curve was plotted using viral RNA of known concentrations. The virus with known titer (7 log10/ml) was serially diluted (spiked) as 1:10 in MDBK cells suspension ($5x10^5$ cells/ ml) The Ct (critical) values (Figure 3-1) with known virus titers were used to plot the

standard curve (Figure 3-2). The standard curve was used to calculate the approximate virus concentration (virus equivalence) in the samples.

Western blot:

To find out whether viral RNA was translated into viral proteins, a Western blot was performed using BVDV infected MDDC lysate. The Western blot assay was done as described previously **(Devireddy and Jones, 1999)** with some modification. The MDDC were infected with ncpBVDV2a-1373 strain of BVDV at a MOI of 6 for 72 hr. After 72 hr, MDDC were washed with PBS and lysed with 200 µl of RIPA buffer (Radioimmunoprecipitation assay buffer*)* containing complete ULTRA tablet containing protease inhibitor (Roch Diagnostic, GmbH, Sandhofen, Germany). The cell lysate were centrifuged at 13,000 rpm for 5 min and supernatant was collected. A 40 µl of cell lysate was mixed with 5µl of 5x loading dye containing beta mercaptoethanol. The cell lysate was denatured by boiling at 100 ºC for 10 minutes. The samples were loaded in 12.5% sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) resolving get. The dual color protein ladder (10-250kD) (Bio-Rad, Hercules, CA, USA) was used as a standard. The proteins were transferred onto a nitrocellulose membrane (Whatman GmbH**,**Hahnestraße, Germany). The nitrocellulose membrane was blocked by 5% skim milk in PBS for 30 minutes at room temperature. The membrane was incubated with anti-BVDV NS5A rabbit polyclonal antibody at a dilution of 1:1000 in PBS containing 1% skim milk at 4 ºC overnight (anti-BVDV NS5A rabbit polyclonal antibody was kindly provided by Dr. Julia F Ridpath**,** Ruminant Diseases and Immunology Research Unit, National Animal Disease Center, USDA, Ames, IA, USA.) After primary antibody

incubation, the membrane was washed three times with PBS containing 0.5% Tween 20. The membranes were incubated with goat anti-rabbit- IRDye 800CW (LI-COR Biosciences, Lincoln, NE, USA) with 1:3000 dilutions in PBS containing 1% skim milk overnight. The specific protein band was visualized by using the Odyssey Imaging system and software (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis:

The virus and viral RNA production in monocytes, MDDC and intermediate stages of MDDC were done at least in three different experiments. The variation in results was calculated by standard deviation at reach time points. The significance difference in virus production between different cell types was calculated with paired T test at 5% level of significance **(Glantz, 2002).**

RESULTS

MDDC yield, morphology and viability

The PBMC yield from 60 ml of blood from the Brown Swiss calves was 1.56 ± 0.5110^{8} compared to $5.36\pm0.73x10^{8}$ PBMC from 60 ml of Holstein Friesian calf blood (Table 2-2). Following incubation for 3 hr on 6-well plates, the adherent cells were harvested using Accutase (Figure 2-1, panels A & D). The harvested cells were confirmed as CD14+ monocytes. A total $1.36\pm0.40x\ 10^{7}$ viable monocytes were obtained from 60 ml blood of Brown Swiss calves compared to $2.833\pm0.35x10^{7}$ viable monocytes, obtained from 60 ml blood of Holstein Friesian calves (Table 2-2). The monocytes were incubated in RPMI medium supplemented with cytokines for their differentiation to MDDC as described above. After 4 days of differentiation, the

monocytes developed dendrites. 24.59±13.74% of monocytes isolated from Brown Swiss exhibited development of dendrites (Figure 2-1, Panel B) while only 11.03±9.70 % of monocytes isolated from Holstein Friesian developed dendrites at day 4 of differentiation (Figure 1, Panel E). At 7 days of differentiation, monocytes became non-adherent and increased in size four to five times with long dendrites.. The monocytes that did not differentiate into MDDC, shrieked and appeared as debris. At day 7 of differentiation, monocytes isolated from Holstein Friesian had more debris (Figure 2-1, Panel C) compared to Brown Swiss (Figure 2-1, Panel F). The differentiated cells were collected by gentle pipetting and further characterized on the basis of morphology and phenotype. A total of $4.2\pm0.72x10^6$ MDDC/collection were harvested from the Brown Swiss calves while in a total of $1.96\pm2.91x10^6$ MDDC/collection were harvested from the Holstein Friesian (Table 2-2).

The comparative study between Holstein Friesian and Brown Swiss indicated that Brown Swiss were able to differentiate superior to MDDC than Holstein Friesian. The monocytes were isolated from eighteen (18) Holstein Friesian and six (6) Brown Swiss calves. Out of the eighteen (18) Holstein Friesian calves used as monocyte donors to differentiate MDDC, one (1) Holstein Friesian calf differentiated higher MDDC in which 25.93% monocytes differentiated to MDDC as compare to other Holstein Friesian calves in which 11.03±9.70% monocytes differentiated into MDDC. The 24.59±13.74% of the total monocytes isolated from the six (6) Brown Swiss calves differentiated into MDDC.

Phenotypic characterization of monocytes and MDDC

At day 0 monocytes were positive for MHCI (96.62±0.50), MHCII (80.58±19.69) and CD14 (16.54±1.49) (Figure 2-2). After 7 days of differentiation, MDDC were stained for MHCI, MHCII, DEC 205, CD86, CD21 or CD14. These MDDC were positive for MHCI (98.58±0.34), MHCII (94.1±2.81), DEC205 (55.97±45.48) and CD86 (77.83±17.83) while they are negative for CD21 (3.42± 0.19) and CD14 (1.025±0.45) (Figure 2-3). During 7 days of differentiation from monocyte to MDDC, the MHCI expression increased ~2.0% (96.62±0.50 to 98.58±0.34) and MHCII expression increased ~17% (80.58±19.69 to 94.1±2.81) while expression of CD14 was significantly ($p > 0.05$) reduced ~94% (16.54±1.49 to 1.025±0.45) (Figure 2-2 and Figure 2-3).

The Effect of BVDV infection on MDDC viability

To determine the effect of BVDV infection on MDDC viability, MDDC were infected with cp BVDV1b-TGAC, ncp BVDV1b-TGAN, ncp BVDV2b-1373 or, ncp BVDV2b-28508-5 strains of BVDV. MDDC were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. along with mock-infected control. The MDDC viability did not change significantly during the course of infection (Figure 2-4). The 100±00%, 99.33±1.15%, 99.33±1.15% and 98.66±1.15% MDDC were viable after 1 hr of infection with ncp BVDV2a-1373, ncp BVDV2a-28508-5, cp BVDV1b-TGAC and ncpBVDV1b-TGAN respectively as compared to 99.33±1.15 % of mock-infected control MDDC (Figure 2-4). At 6 hr p.i., 97.33±1.15%, 97.33±1.15%, 98.00±00%, 96.66±1.15% MDDC were viable following infection with ncpBVDV2a-1373, ncpBVDV2a-28508-5, TGAC and TGAN strains of BVDV respectively as compared to 98.66±1.15% in mock-infected control

MDDC at that time point (Figure 2-4). Viability was still high at 12 hr p.i. with 96.66±1.15%, 96.00±2%, 96.66±1.15%, and 95.33±2.309% MDDC following infection with ncpBVDV2a-1373, ncp BVDV2a-28508-5, cp BVDV1b-TGAC and ncp BVDV1b-ncpTGAN strains respectively as compared to 97.33±1.15% in mock-infected control MDDC at 12 hr (Figure 2-4).

At 24 hr p.i. with ncpBVDV2a-1373, ncp BVDV2a-28508-5, cp BVDV1b-TGAC or ncp BVDV1b-TGAN strains 96.00±2.30%, 96.66±2.30%, 96.00±1.15%, and 94.66±1.15% MDDC respectively were viable as compared to 97.33±1.15% of mock-infected control MDDC at 24 hr (Figure 2-4). The 96.00±3.4%, 96.66±2.30%, 94.00±3.46% and 94.66±1.15% MDDC were viable after 48 hr p.i. with ncpBVDV2a-1373, ncpBVDV2a-28508-5, ncpBVDV1b-TGAC and ncpBVDV1b-TGAN strains of BVDV respectively as compared to 96.66±1.15% in mock-infected control MDDC at that time point. The 94.66±3.055%, 94±2%, 94±3.46% and 94±2 % cells were viable 72 hr p.i. with ncpBVDV2a 1373, ncpBVDV2a 28508-5, cpBVDV1b TGAC or ncpBVDV1b-TGAN infection respectively as compared to 96.66±1.15% viability in mock-infected MDDC (Figures 2-4 and Table 2-3). During this period MDDC infected with cp TGAC strain of BVDV didn't produce any cytopathic effect (Figure 2-5).

Infectious BVDV production in MDDC, Monocytes or in MDBK cells:

The purpose of this experiment was to investigate whether MDDC supported BVDV replication and to compare BVDV virus production in MDDC with BVDV virus production in monocytes or in MDBK cells. The BVDV virus production was measured at 1hr, 6 hr, 12hr, 24 hr, 48hr, 72 hr or 96 hr p.i. BVDV-infected MDBK cells were used

as a positive BVDV replication control at each time point. There was no infectious BVDV produced by MDDC at any time point from 1 hr to 96 hr p.i. The inoculum virus of MDDC declined from 5.698 log 10/ml to zero within 96 hr p.i. in all BVDV strains used in the study (Figures 2-8, 2-9, 2-10, 2-11).

The MDDC infected with cp BVDV1b-TGAC strain of BVDV did not produced any infectious virus while its precursor, monocyte became infected and produced infectious virus as early as 24 hr p.i. Monocytes produced the cp BVDV1b-TGAC strain at a titer of 2.70±00 log 10/ ml at 24 hr p.i. The supernatant of monocyte had a virus titer of 3.20±0.70 log 10/ml at same time point (24 hr p.i.). The virus titer of cp BVDV1b-TGAC strain in monocytes peaked at 4.698±00 log10/ml at 72 hr p.i. and declined to 4.198±0.70 log10/ml at 96 hr p.i. The virus titer of cp BVDV1b-TGAC in monocyte supernatant reached its peak at 4.698±00 log10/ml at 48 hr p.i. and maintained this titer up to 96 hr p.i.

The virus production of cp BVDV1b-TGAC stains in MDBK cell started as early as 12 hr p.i., which was 12 hr earlier than in monocytes. At 12 hr p.i., MDBK cells had a virus titer of 2.70±00 log 10/ ml that peaked at 72 hr p.i. as 6.20±0.70 log 10/ ml and maintained same up to 96 hr p.i. The production of cp BVDV1b-TGAC in MDBK supernatant began at 24 hr p.i. with a titer of 4.20± 0.70 log10/ml and peaked at 6.70± 0.00 log10/ml at 96 hr p.i. (Figure 2-8).

Similar to its homologues, the infectious virus of ncp BVDV1b TGAN strain of BVDV was not produced by MDDC while monocytes produced ncp BVDV1b-TGAN. In monocytes, the ncp BVDV1b-TGAN virus was detected as early as 24 hr p.i. as 2.20±

0.70 log 10/ml that increased and reached to its peak at 72 hr p.i. as 4.70 ± 0. 0 log 10/ml and declined at 96 hr p.i. (4.20 ± 0.70 log 10/ml) in monocytes. The infectious ncp BVDV1b-TGAN released in monocyte supernatant as early as 24 hr p.i. At 24 hr p.i. ncp BVDV1b-TGAN titer was detected as 3.20± 0.70 log 10/ml that reached to its peak as 4.70± 0.00 log 10/ml at 48 hr p.i. and maintained it up to 96 hr p.i. (Figure 2-9)

In MDBK cells the ncp BVDV1b-TGAN virus was detected as early as 12 hr p.i. with titer of 2.7-± 0.00log 10/ml and reached to its peak at 96 hr p.i. (6.20± 0.70 log 10/ml). In MDBK cell supernatant, the ncp BVDV1b-TGAN virus was detected as early as 12 hr p.i. with titer of 0.85±1.2 log10/ ml and reached to its peak at 96 hr p.i. as 6.70±00 log 10/ml (Figure 2-9).

The MDDC also not supported the production of typical virulent ncpBVDV2a-28508-5 strain of BVDV while monocytes start producing ncpBVDV2a 28508-5 as early as 24 hr p.i. The monocyte and its supernatant revealed the titer of ncpBVDV2a-28508-5 as 1.70±00 log10/ ml and 2.20±71 log10/ ml respectively at 24 hr p.i. The virus titer of ncpBVDV2a-28508-5 reached to its peak in monocytes and its supernatant as 2.70±00 log10/ ml and 5.70±00 log10/ ml at 72 hr p.i. than declined at 96 hr p.i. as 2.20±0.70 log10/ ml and 4.70±00 log10/ ml respectively (Figure 2-10).

The MDBK cells start producing ncpBVDV2a-28508-5 as early as 24 hr p.i. that was 12 hr later than any other viral strains used in the study. The virus titer of ncpBVDV2a-28508-5 was observed in MDBK cells as 1.35±1.9 log10/ ml at 24 hr p.i. The virus titer of ncpBVDV2a-28508-5 increased and reached to its peak, in both MDBK

cells and its supernatant at 96 hr p.i. as 6.70±00 log10/ ml and 6.70±70 log10/ ml respectively (Figure 2-10).

The MDDC infected with high virulent ncpBVDV2a-1373 strain of BVDV did not produce any infectious virus while its precursor, monocyte became infected and produced infectious virus. The monocytes start producing ncpBVDV2a-1373 strain of BVDV at 24 hr p.i. The titer of ncpBVDV2a-1373 strain of BVDV in monocytes revealed as 2.20± 0.70 log 10/ml at 24 hr p.i. while titer of ncpBVDV2a-1373 strain of BVDV in supernatant of monocytes was found as 2.70 ±00 log 10/ ml at 24 hr p.i. The titer of ncpBVDV2a-373 strain of BVDV reached to peak as 3.70±00 log 10/ ml at 48 hr p.i. in both monocytes and its supernatant and maintained up to 72 hr p.i. than declined in monocytes as 2.70±00 log 10/ ml at 96 hr p.i. (Figure 2-11).

The ncpBVDV2a-1373 strain start producing infectious virus in MDBK cells 12 hr earlier than monocytes. The MDBK cells showed the ncpBVDV2a-1373 virus production at 12 hr p.i. with titer of 2.20± 0.70 log 10/ml while virus released in supernatant 12 hr later and detected as early as 24 hr p.i. with titer of 4.20± 0.70 log 10/ml. The virus titer of ncpBVDV2a-1373 strain of BVDV reached to its peak in MDBK cells as 6.20± 0.70 log 10/ml at 72 hr p.i. that maintained up to 96 hr p.i. The peak titer of ncpBVDV2a-1373 strain of BVDV in MDBK cells supernatant was observed 12 hr later than its cell at 96 hr p.i. as 7.20±0.70 log 10/ml (Figure 2-11).

BHV1 production in MDDC:

The experiments described above established that BVDV was produced in monocytes (the precursor of MDDC) while fully differentiated MDDC did not supported

the production of infectious BVDV. To deterimine if this inhibition in MDDC was specific for BVDV or a generalized property of MDDC, the MDDC were infected with the Cooper strain of BHV1 and virus production determined. BHV1 was choosed as a positive control virus because earlier studies showed that MDM (monocyte-derived macrophage) supported BHV1 production while MDM did not produce any infectious BVDV following BVDV infection **(Elmowalid, 2003).**

The MDDC were infected with the Cooper strain of BHV1 at a MOI of 6. After 1 (one) hr adsorption, MDDC were washed three times by suspending them in PBS and centrifugation at 1700 rpm for 15 min at 4°C. Finally, MDDC were suspended in RPMI 1640 medium supplemented with 10% FBS, 1mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 μg/ml) to achieve final concentration of 5x 10^5cells/ ml. One ml of this cell suspension was added to each well of a 24-well plate. The cells from one well were collected at a time point. The cells and supernatant were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr p.i. The cells were examined for their viability and BHV1 production. The cell viability was examined by trypan blue exclusion assay as described in chapter 2. The cell viability was reduced following BHV1 infection from 99.33±0.58% at 1 hr p.i. to 78.33±0.58% at 96 hr p.i. as compare to mock-infected MDDC as 99.66±0.577% at 1 hr to 94.33±0.577% at 96 hr p.i. (Table 2-4, Figure 2-12).

The MDDC produced infectious BHV1 as early as 12 hr p.i. At 12 hr p.i., MDDC BHV1 virus titer was 2.03±0.58 log 10/ml while MDDC supernatant had a BHV1 titer as 0.57±0.98 log 10/ml (Figure 2-13).The BHV1 titer increased during the course of infection and peaked at 48 hr p.i. in both cell and supernatant and maintained up to 96 hr

p.i. At 48 hr p.i., the MDDC BHV1 virus titer was 4.70±0.00 log 10/ml while MDDC supernatant had a BHV1 titer of 7.70±0.00 log 10/ml (Figure 2-13). The MDBK cells produced BHV1 as early as 6 hr p.i. The MDBK BHV1 titer was 6.36±0.58 log 10/ml at 6 hr p.i. and increased to 7.70±0.00 log 10/ml at 12 hr p.i. and maintained this titer up to 24 hr p.i. The BHV1 titer declined in MDBK cells at 48 hr p.i. as 7.37±0.58 log 10/ml, at 72 hr p.i. as 6.70±0.00 log 10/ml and 6.03±0.58 log 10/ml (Figure 2-13).

BHV1 virus appeared in MDBK supernatant as early as 6 hr p.i. The BHV1 virus titer in MDBK supernatant was 5.03±0.58 log 10/ml at 6 hr p.i. and increased to 7.70±0.00 log 10/ml at 12 hr p.i. (Figure 2-13). This level of virus was maintained up to 72 hr p.i. The virus titer of BHV1 declined in MDBK cell supernatant at 96 hr p.i. to 7.36±0.58 log 10/ml (Figure 2-13).

The decline in BHV1 titer in MDBK cells within 24 hr p.i. was due to death of MDBK cells as there were no viable cells at 24 hr p.i. using trypan blue exclusion assay (data not shown).

Replication of BVDV in intermediate stages of MDDC development:

Since BVDV replicated in monocytes but not in monocyte-derived dendritic cell (MDDC), the stage of MDDC differentiation and its effect on BVDV replication was examined. The monocytes were cultured as described above for MDDC development. The cytokine-treated monocytes were harvested at 48 hr (2 days), 72 hr (3 days), 96 hr (4 days) or 120 hr (5 days) of differentiation. These intermediate stages of MDDC were infected with BVDV ncpBVDV2a-1373 strain at a 6 MOI of infection. Cells were collected at 24 and 48 hr p.i. for each differentiated stage and just the cells were tested for

virus production. Virus titer was determined in intermediate stages of MDDC as described above. Virus production decreased with the length of time of differentiation. The intermediate stage of MDDC infected at 48 hr of differentiation produced virus with a titer of 3.70±00 log10/ml at 24 hr p.i. and the virus titer was reduced one log (2.70±00 log10/ml) at 48 hr p.i. The intermediate stage of MDDC infected at 72 hr of differentiation, produced virus with a titer of 2.20±0.71 log10/ml at 24 hr p.i. that was reduced to 1.70±00 log10/ml at 48 hr p.i. The intermediate stage of MDDC infected at 96 hr of differentiation produced virus at a titer of 1.70±00 log10/ml at 24 hr p.i. that was reduced to 0.85±1.2 log10/ml at 48 hr p.i. The intermediate stage of MDDC infected at 120 hr of differentiation did not produce any virus at either 24 hr p.i. or 48 hr p.i. (Figure 2-14), indicating that MDDC loss the ability to produce virus between 96 and 120 hr of differentiation. Interestingly during this period from 96-120 hr of differentiation, when the cells lost the ability to produce infectious virus the MDDC showed significantly morphological changes. The MDDC increased 4-5 times in size and had extensive development of dendrites.

Replication of BVDV viral RNA in MDDC or in MDBK cells:

Since MDDC were unable to produce infectious BVDV, to determine whether BVDV replicates in MDDC, the MDDC were infected with 4 strains of BVDV including high virulent ncpBVDV2a-1373, typical virulent ncpBVDV2a-28508-5 strains of BVDV and a homologous pair of ncp and cp type viruses, ncpBVDV1b-TGAN and cpBVDV1b-TGAC, recovered from an animal that died of mucosal disease.

Briefly, MDDC were infected with virus at 6 MOI of infection with one of BVDV strains. Cells were collected at 1 hr, 6 hr, 12 hr, 72 hr, 96 hr, 144 hr, 168 hr or 192 hr p.i. Cells were washed with PBS and viral RNA was extracted as described above. The MDBK cells infected with virus at 6 MOI with one of each of the BVDV strains was used as controls. The ncpBVDV2a-1373 strain of BVDV with known titer (7 log10/ml) was serially diluted as 1 in 10 in MDBK cells suspension. The viral RNA was extracted from known concentrated viruses and quantified using qRTPCR. The Ct (critical) values with known virus titers were used to plot the standard curve. The standard curve was used to calculate the approximate virus concentration (virus equivalence) in the samples (Figure 2-6, Figure 2-7).

Viral RNA was extracted from MDDC following BVDV infection and quantified to determine if any viral RNA replicated in the MDDC.

The results indicated that the BVDV viral RNA replicated in MDDC. The kinetics of viral RNA production along with the amount of viral RNA was significant different between different viral strains.

cp BVDV1b-TGAC strain viral RNA replicated in MDDC as early as 1 hr p.i. with virus equivalence 3.70±00 log10/ml. The TGAC viral RNA concentrations increased up to 144 hr p.i. and reach its peak at 7.20±0.71 log10/ml virus equivalence at 144 hr p.i. The cp BVDV1b-TGAC viral RNA concentration declined at 168 hr and 192 hr p.i. to 5.70±0.00 log10/ml virus equivalence and 4.70±0.71 log10/ml virus equivalence respectively. The MDBK cells also replicated TGAC viral RNA as early as 1 hr p.i. The cp BVDV1b-TGAC viral RNA in MDBK cells was 4.70±0.00 log10/ml virus

equivalence at 1 hr p.i. and was 7.70±0.00 log10/ml virus equivalence at 72 hr p.i. cp BVDV1b-TGAC viral RNA in MDBK cells start declining at 144 hr p.i. The cp BVDV1b-TGAC viral RNA in MDBK cells was 7.198±0.707 log10/ml virus equivalence at 144 hr p.i. and declined 6 logs to 0.5±0.70 log10/ml virus equivalence at 168 hr p.i. (Figure 2-15).

The cp BVDV1b TGAC ncp homolog pair, ncp BVDV1b-TGAN started replicating viral RNA in MDDC 12 hr p.i. with RNA concentration as 3.70±0.00 log10/ml virus equivalence. The ncpBVDV1b-TGAN viral concentration increased half log at 48 hr p.i. (4.20±0.71 log10/ml) and one log at 72 hr p.i. as 4.70±0.00 log10/ml virus equivalence that was maintained to 96 hr p.i. than declined one log at 144 hr p.i. as 3.70±0.00 log10/ml virus equivalence. The The ncpBVDV1b-TGAN viral concentration further declined one log at 192 hr p.i. as detected as 3.70±0.00 log10/ml virus equivalence (Figure 2-16).

In MDBK cell, the ncpBVDV1b-TGAN viral RNA was detected as early as at 1 hr p.i. as 4.70±0.00 log10/ml virus equivalence. The The ncpBVDV1b-TGAN viral concentration increased with course of time and reached 5.20±0.71 log10/ml virus equivalence at 48 hr p.i. and 6.70±0.00 log10/ml virus equivalence at 72 hr p.i. and declined at 96 hr p.i. as 6.2-±0.71 log10/ml virus equivalence. The TGAN viral RNA further declined at 144 hr and 168 hr p.i. At 144 hr and 168 hr p.i. the TGAN viral RNA was 5.70±0.00 log10/ml and 3.70±0.00 log10/ml virus equivalence respectively (Figure 2-16).

The typical virulent ncp BVDV2a-28508-5 strain of BVDV started replicating viral RNA in MDDC as early as 6 hr p.i. The 28508-5 viral RNA was detected at 1.70±0.00 log10/ml virus equivalence at 6 hr p.i. that increased one log at 12 hr p.i. (2.70±0.00 log10/ml virus equivalence) and an additional log at 48 hrs p.i. (3.698±0.00 log10/ml virus equivalence) and remain same at 72 hr p.i. The 28508-5 viral RNA concentration was maintained at 96 hr p.i. (3.70±0.00 log10/ml virus equivalence) while no ncp BVDV2a-28508-5 28508-5 viral RNA was detected after 144 hr in MDDC (Figure 2-17).

The viral RNA of ncp BVDV2a-28508-5 was detected in MDBK cells as early as 1 hr p.i. At 1 hr p.i.. The 28508-5 viral RNA was 2.70±0.00 log10/ml virus equivalence that increased one log to 3.70±0.00 log10/ml virus equivalence and maintained this level at 12 hr p.i. The ncp BVDV2a-28508-5 viral RNA increased approximate two logs from 12 hr p.i. (3.70±0.00 log10/ml virus equivalence) to 48 hr p.i. (5.70±0.00 log10/ml virus equivalence) and one approximate log further at 72 hr p.i. (6.20±0.70 log10/ml virus equivalence) . The viral RNA concentration declined approximately three logs at 144 hr p.i. (3.70±0.00 log10/ml virus equivalence) and further one log at 168 hr p.i. (2.70±0.00 log10/ml virus equivalence) (Figure 2-17).

The high virulent ncp BVDV2a-1373 strain of BVDV started replicating viral RNA at 24 hr p.i. (3.70±0.00 log10/ml virus equivalence) and reached 5.70±0.00 log10/ml virus equivalence at 144 hr p.i. and declined one log (4.70±0.00 log10/ml virus equivalence) at 192 hr p.i. (Figure 2-18). As compare to MDDC, the ncp BVDV2a-1373 strain of BVDV started replicating viral RNA 23 hr earlier in MDBK cells. In MDBK

cells, the viral RNA of ncp BVDV2a-1373 strain of BVDV was detected at 1 hr p.i. (4.70±0.00 log10/ml virus equivalence) that increased during the course of infection and reached 6.70±0.00 log10/ml virus equivalence 48 hr p.i. The viral RNA of the ncp BVDV2a-1373 strain of BVDV start declining in MDBK cells at 96 hr p.i. (6.20±0.70 log10/ml virus equivalence) and declined to 5.70±0.00 log10/ml virus equivalence at 144 hr p.i. and at 168 hr p.i. and declined one log further at 192 hr p.i. (4.70±0.00 log10/ml virus equivalence) (Figure 2-18). Presence of viral RNA and its replication indicated that MDDC became infected with all viral strains of BVDV used in the study.

BVDV viral RNA replication of 1373 in MDDC, in monocytes or in MDBK cells:

Among the three cells types, the monocytes and MDBK cells began replicating viral RNA at 1 hr p.i. At 1 hr p.i. MDBK cell revealed highest viral RNA concentration as (4.70±0.00 log10/ml virus equivalence) that was approximately three (3) logs higher than viral RNA in monocytes at same time point (1.35±1.90 log10/ml virus equivalence). In monocytes, the viral RNA peaked at 24 hr p.i. as 5.20±0.70 log10/ml virus equivalence that was maintained up to 48 hr p.i. and declined approximately two logs at 72 hr p.i. (3.70±1.41 log10/ml virus equivalence). The viral RNA in monocytes further reduced to 1.00±1.414 log10/ml virus equivalence at 96 hr p.i. while no viral RNA was detected in monocytes at 168 hr p.i.

In MDBK cells, the viral RNA reached the peak at 48 hr p.i. at 6.70±0.00 log10/ml virus equivalence. In MDBK cells, viral RNA began declining at 96 hr p.i. (6.20±0.70 log10/ml virus equivalence) and declined 2 logs to 4.70±0.00 log10/ml virus equivalence at 168 hr p.i. (Figure 2-19). In MDDC, the viral RNA start replicating at 24

hr p.i. (3.70±0.00 log10/ml virus equivalence) and declined at 192 hr p.i. with viral RNA as 4.70±0.00 log10/ml virus equivalence.

This study indicated that BVDV viral RNA start replicating in monocytes and MDBK cell much earlier (1 hr p.i.) than MDDC (24 hr p.i.). The viral RNA in monocyte remained lower at all the time point as compared to MDBK cells. In monocytes the replication of viral RNA completely ceased by 168 hr p.i. These RNA findings were in agreement with the infectious virus production by monocytes and MDBK cells. MDBK cells produced more virus than monocytes at all time points (Figure 2-8, Figure 2-9, Figure 2-10 and Figure 2-11). BVDV viral replication in MDDC started later (24 hr p.i.) than monocytes and MDBK cells and viral RNA levels remained higher at 192 hr p.i. than in monocytes (Figure 2-19).

Detection of UV inactivated 1373 strain of BVDV in MDDC:

The UV inactivated ncpBVDV2a-1373 strain of BVDV did not replicate in MDDC. The Ct (critical value) of qRTPCR for BVDV RNA in MDDC infected with UV inactivated ncpBVDV2a-1373 strain of BVDV ranged from 35.37 to 36.81 from 1 hr p.i. to 96 hr p.i. [1 hr p.i.(35.53), 6 hr p.i. (36.81), 12 hr p.i. (35.830, 24 hr p.i.(35.37), 72 hr p.i.(36.24) and 96 hr p.i. (36.29)]. These Ct values were near zero (0.0 TCID50/ml) as determined by the standard curve (Figure 2-6 and Figure 2-7). These results indicated that the input virus RNA alone did not produce Ct values with higher viral titers measured during the course of infection with the four strains of BVDV.

Presence of NS5A protein of BVDV in MDDC:

The BVDV replicated viral RNA in MDDC but did not produce infectious virus. To determine whether viral RNA was translated into viral proteins, a Western blot was performed. The Western blot was carried out using ncpBVDV2a-1373-infected MDDC lysate. The MDDC were infected with ncpBVDV2a-1373 strain of BVDV. Infected MDDC were washed with cold PBS and lysed by RIPA buffer (Radioimmunoprecipitation assay buffer) containing protease inhibitor. MDDC lysate revealed the presence of NS5A viral protein with size of 56 kD (Figure 2-20), indicating that MDDC did produce viral proteins but do not release infectious virus.

DISCUSSION

The key findings of this chapter were the breed differences in the MDDC development and restricted ability of MDDC to produce infectious BVDV. All Brown Swiss calves used in the study produced higher numbers of MDDC as compared to Holstein Friesian. In case of Brown Swiss, 24.59±13.74% of the monocytes differentiated into MDDC while only 11.03±9.70% monocytes isolated from Holstein Friesian differentiated into MDDC. No cytopathic effect was noticed in MDDC infected with cp BVDV1b-TGAC. In previous study, cp BVDV on DC viability was observed in an *in vivo* trial in 2.5 to 7 month old Holstein Friesian PI calves. Super infection of these PI calves with cp BVDV did not affect the number or the morphology of dendritic cells in intestinal lymphoid follicles. Interestingly these dendritic cells were surrounded by apoptotic lymphocytes, indicating that cp BVDV cause apoptosis in lymphocytes but not in DC **(Teichmann et al., 2000).**

The result revealed that MDDC became infected but did not support the production of infectious BVDV. The progenitor cell of the MDDC, the monocyte, became infected with BVDV and produced infectious virus while the intermediate stages of MDDC produced virus at levels that were proportional to the length of time they had been cultured to become MDDC. No infectious virus was produced in fully differentiated MDDC while viral RNA replicated in MDDC. The kinetics of viral RNA production varied between the different viral strains in MDDC. The high virulent ncpBVDV2a-1373 strain began replicating viral RNA later (24 hr p.i.) than other strains and accumulated viral RNA for longer duration (192 hr p.i.). The amount of viral RNA of ncpBVDV2a-1373 strain of BVDV accumulated more in MDDC as compared to levels of viral RNA of ncpBVDV2a-28508 strain of BVDV. The typical virulent ncpBVDV2a-28508-5 strain began replicating viral RNA at an early stage of infection (6 hr p.i.) and viral replication declined at 96 hr p.i. while there was no viral RNA present by 120 hr unlike the three other strains where viral RNA could still be detected at 192 hr when the study ended. The replication of viral RNA of cp BVDV1b-TGAC strain began at 1 hr p.i. and peaked at 72 hr p.i. and declined at 192 hr p.i. The ncp BVDV1b-TGAN strain of BVDV began replicating viral RNA in MDDC at 12 hr p.i. and reached its peak at 72 hr p.i. then began declining at 144 hr p.i.

Among all strains used in the study, the cp BVDV1b-TGAC strain of BVDV start replicating viral RNA earlier than any of ncp BVDV strains. The cp TGAC strain of BVDV start replicating viral RNA at 1 hr p.i. The earlier study revealed that cp-BVDV1b-TGAC significantly up regulate the MHCI and CD86 expression in MDDC at

1 hr p.i. The up regulation of MHCI and CD86 with cp BVDV1b-TGAC viral replication may be reason of effective immune response mounted by cp strain of BVDV in live modified vaccines. The cp BVDV1b-TGAC viral RNA replication showed a direct correlation with MHCI, MHCII and CD86 expression. The cp BVDV1b-TGAC viral RNA increased from 3.70±0.00 log10/ml virus equivalence at 1 hr p.i. to 5.70±0.00 log10/ml virus equivalence at 72 hr p.i. During this period the expression of MHCI, MHCII and CD86 increased approximately 20% (133.51±11.88% to 158.17±52.43%) 20% (119.16±9.33% to 156.83±54.48%) and 150% (111.40±3.55% to 261.22±162.00%) respectively as compare to time point control. Whereas the homologues ncp BVDV1b-TGAN strain of BVDV showed indirect correlation between RNA replication and MHCI, MHCII and CD86 expression. The ncp BVDV1b-TGAN viral RNA increased from zero to 4.70±0.00 log10/ml virus equivalence within 72 hr p.i. During this period the expression of MHCI, MHCII and CD86 reduced approximately 15% (80.94±9.14% to 64.15±9.62%) 10% (90.02.16±0.10% to 79.33±19.13%) and 25% (99.48±6.13% to 72.64±1.38%) respectively as compare to time point control. The other ncp BVDV (BVDV2a-1373 and BVDV2a-28508-5) used in the study also showed the indirect correlation between RNA replication and MHCI, MHCII and CD86 expression. The difference in correlation may be due to differential effect of these biotypes in cell culture **(Zhang et al., 1996)** or**,** difference in induction of cytokines **(Diderholm and Dinter 1966; Adler et al., 1997)** or may be due to their viral protein processing where cp BVDV strains cleave the NS23 viral protein into two proteins: NS2 (p54), and NS3 (p80) while it is not seen in ncp BVDV strains **(Brownlie, 1990).**

One of the issues in using PCR based assays is high sensitivity that does not translate to understanding if the virus is biologically functional.

The BVDV viral RNA replication was compared between monocyte and MDDC using the high virulent ncp BVDV2a-1373 strain of BVDV. The RNA replication in monocytes started earlier than MDDC. The monocytes start producing infectious BVDV as early as 24 hr p.i. and BVDV viral RNA replicates as earlier as 1 hr p.i. The MDDC did not produce infectious BVDV at any time point while viral RNA replicated as early as 72 hr p.i. for ncp BVDV2a 1373 strain of BVDV. Monocyte accumulated less amount of viral RNA than MDDC whereas monocytes produced infectious virus. These findings indicated that MDDC either do not translate the viral proteins or there is hindrance in viral assembly or release in MDDC. The earlier statement could not be true because the BVDV viral RNA encode a polyprotein that is cleaved into various viral proteins including NS5B. The NS5B viral protein (p75) acts as RNA-dependent-RNA polymerase and replicates the viral RNA. The increasing amount of viral RNA at different time points of MDDC indicated the presence of the NS5B viral protein and BVDV RNA translation in MDDC.

The lack of infectious virus production by MDDC was different from previous results in bovine MDDC (**Glew et al., 2003**). In a previous study, a homologous pair of BVDV ncp and cp viruses, Pe515ncp and Pe515cp, replicated and produced infectious virus in MDDC. The difference in results may due to use of different monocyte isolation and MDDC culturing methods. In the previous studies, the MDDC were CD14+ adherent cells **(Glew et al., 2003; Werling et al., 1999)** while in the current study the MDDC were

non-adherent CD14- cells. The cells in the previous studies had a more monocyte pheonotype and may have represented a more intermediate MDDC phenotype such as was observed at 2-4 days of monocyte to MDDC differentiation observed in this study.

The adherent CD14+ cells which have phenotypical resemblance to monocytes or intermediate MDDC phenotype such as was observed at 2-4 days produced infectious virus. Such as in a study using BVDV Suwa Ncp strain, monocyte-derived macrophage replicated the BVDV RNA and produced infectious virus **(Schweizer et al., 2006).** Infection studies with hepatitis C virus (HCV), another virus of flaviviridae family were done in human MDDC. Human MDDC, infected with HCV JFH1 strain, neither supported HCV RNA replication nor antigen production **(Ebihara et al., 2008).** The diffrence in results may be due to diffrence in cell type and virus used. The HCV replication is restricted to human hepatocytes or human PBMCs wheras BVDV can infecte and replicate in almost in all bovine cells specially epithelia cells. The HCV viral RNA was detected in peripheral blood DC **(Goutagny et al., 2003)** but HCV infected DC did not replicate HCV *in vitro*. The J6/JFH (a chimeric HCV of genotype 2a) did not replicate in B or T lymphocytes, monocytes, macrophages, or dendritic cells while it replicated and produced infectious virus in Huh-7.5 cells, a liver cell line **(Marukian et al., 2008).**

Another study was conducted to detect non-structural BVDV protein, p80 (NS3) and the structural BVDV protein, gp53 (E2) in peripheral blood mononuclear cells (PBMC) isolated from viremic animals infected with ncp pe515 strain of BVDV. This study revealed that monocytes expressed the highest level of NS3 (p80) followed by T

cells, B cells and γδ T cells **(Sopp et al., 1994)**. This study also showed that the bovine monocyte became infected with BVDV and produced infectious virus which is in agreement with the current findings. The monocytes produced infectious BVDV up to 48 hr to 72 hr p.i. The maximum BVDV virus titer was observed in monocytes at 72 hr p.i. followed by reduction in virus titer at 96 hr p.i.

In this study, BVDV was produced by monocytes, the MDDC precursor, but not by fully differentiated MDDC. To find out at which stage of MDDC differentiation, MDDC lose the capacity to produce infections virus, the monocytes were harvested at 48 hr (2 days), 72 hr (3 days), 96 hr (4 days) or 120 hr (5 days) of differentiation to MDDC. These intermediate stages of MDDC were infected with high virulent BVDV2a-1373 strain of BVDV at a 6 MOI of infection. Cells were collected at 24 and 48 hr p.i. for each differentiation stage. Virus titer was determined in intermediate stages of MDDC. Results showed that virus production decreased with the length of time of differentiation. MDDC completely lost the ability to produce virus at 120 hr (5 days) of differentiation. This is the time when monocyte start changing morphologically to MDDC with development of dendrites and increasing in size (4-5 times than initial).

The result of current study indicated that BVDV infects MDDC, replicate viral RNA and translate viral protein but is not release infectious virus from MDDC. The mechanism/s that prevents either packaging or release of infectious virus need to be explored.

Result showed that MDDC has higher amount of viral RNA as compare to monocytes or MDBK cells at 168 hr or 192 hr p.i. in all the BVDV strains used in the

study except typical virulent ncp BVDV2a-28508-5. This viral RNA may be translated into viral proteins and finally infectious virus after interaction of MDDC to T cell or when MDDC migrate to different microenvironment.

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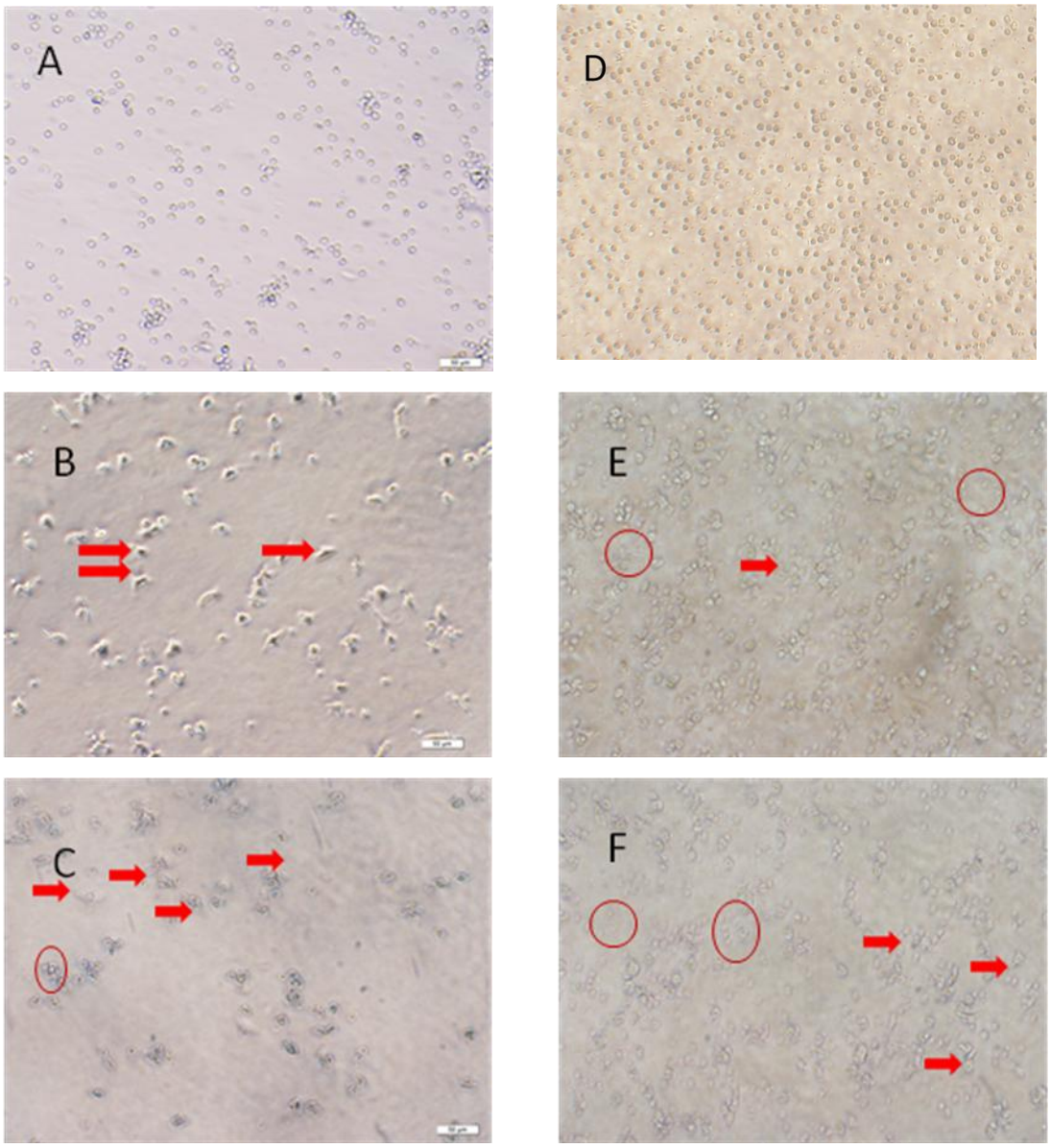

Figure 2-1. Effect of breeds in differentiation of MDDC. Panel A-C: Showing the differentiation of monocyte to MDDC isolated from Brown Swiss at day 0, A). day 4 B) and Day 7 C). Panel D-F: Representing the differentiation of monocytes to MDDC isolated from Holstein Friesian at day 0, D). day 4 E) and day 7 F). At day 4 and 7 cells showing dendrites (with red arrows) and debris (red circle). Most of cells were either loosely attached or floating in the medium.

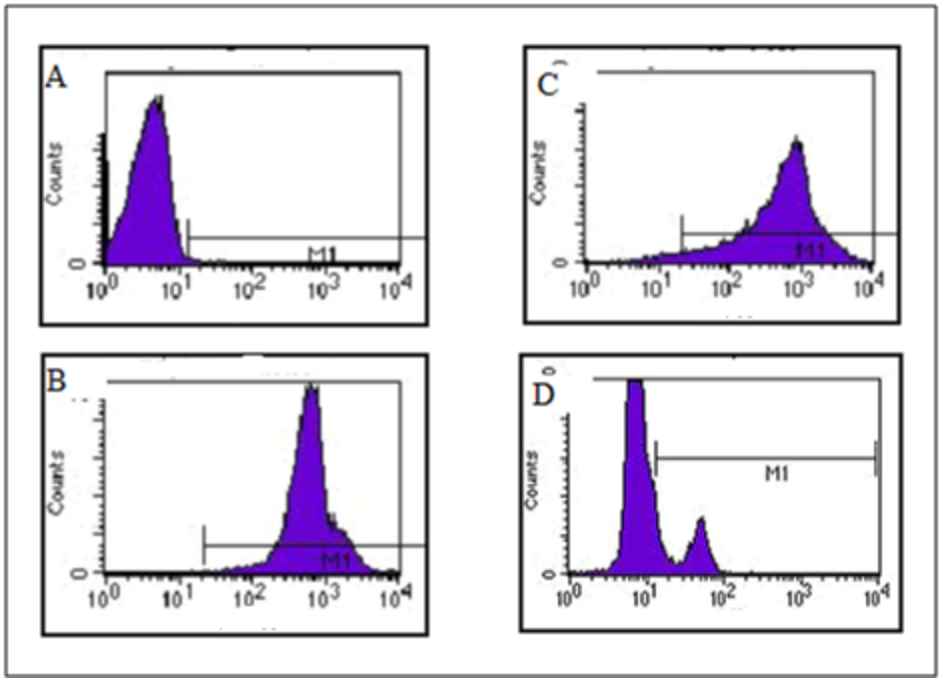

Figure 2-2. Phenotype of adherent bovine monocytes. The adherent monocytes were stained with MHCI, MHCII or CD14 primary antibodies followed by FITC-labeled secondary antibody. A) Secondary antibody control cells showing 2.034±0.67% gated cells; B) Cells showing MHCI expression with 96.62±0.5%; C) Cells showing MHCII expression with 80.58± 19.69% expression; D) Cells showing CD14 expression 16.54±1.49%.

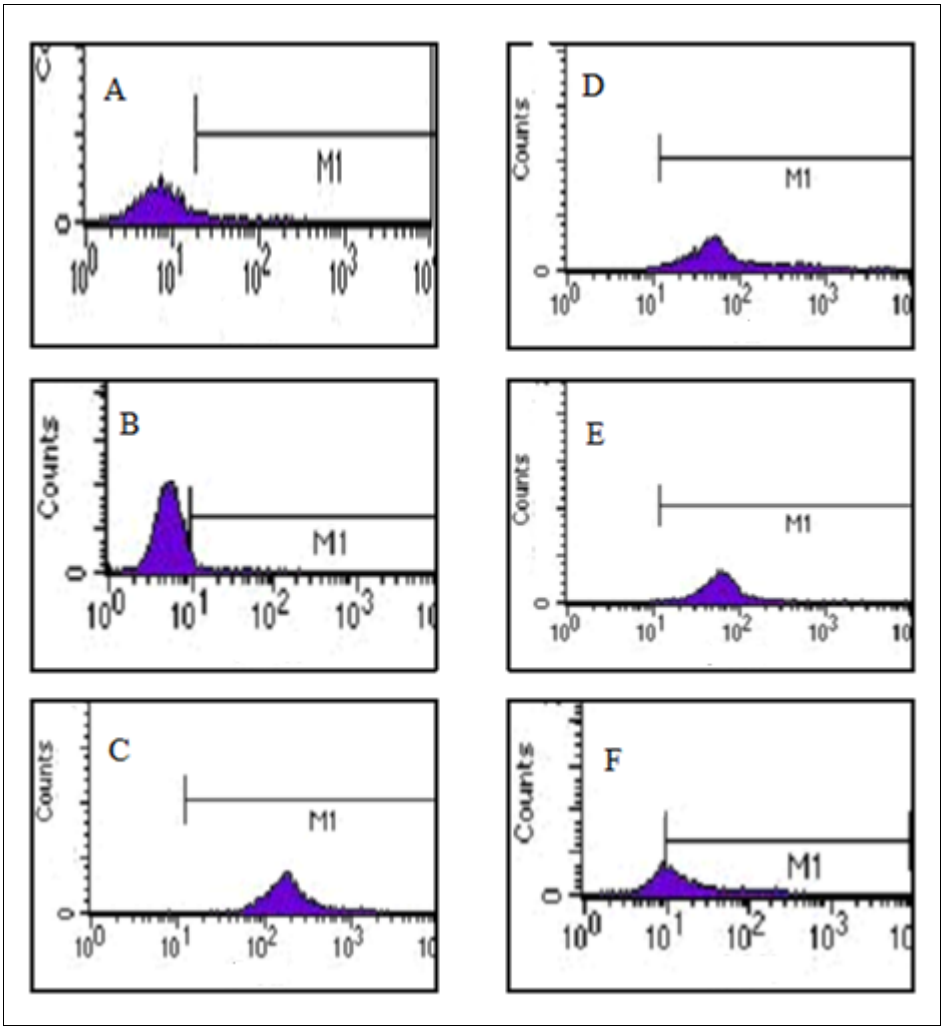

Figure 2-3.Phenotype of bovine monocyte-derived dendritic cell (MDDC). The MDDC at seven days of differentiation were stained for CD14, DEC205, MHCI, MHCII or CD86 A) MDCC CD14 expression was 1.025 ± 0.45%; B) MDCC CD21 expression was 3.42±0.19%; C) MDCC MHCI expression was 98.58± 0.34%; D) MDCC MHCII expression with 94.1±2.81%; E) MDCC CD86 expression was 77.83±17.83%; F) MDCC DEC205 expression was 55.97±45.48%.

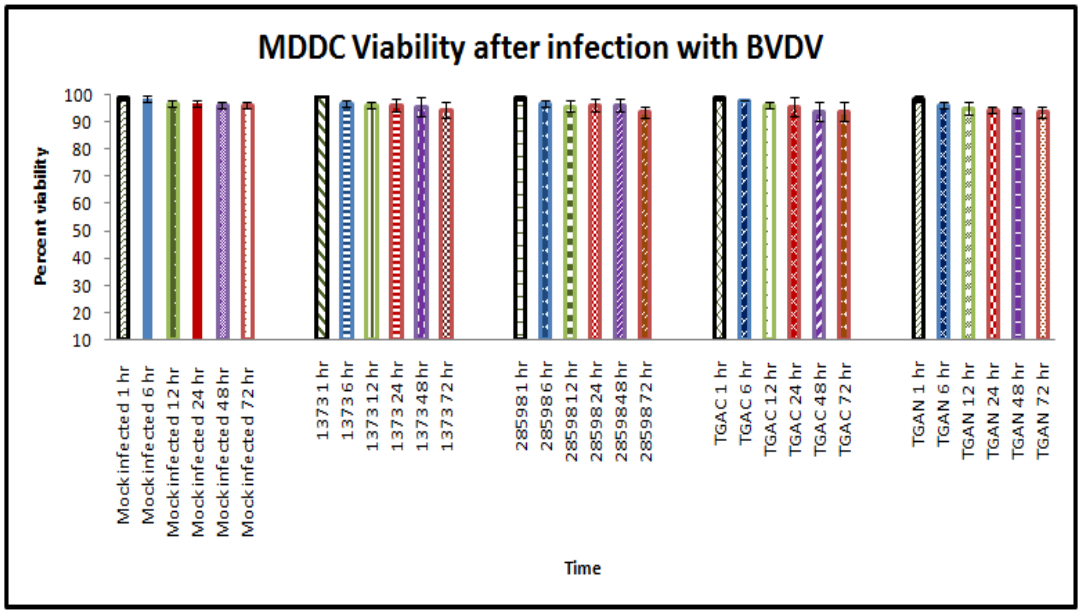

Figure 2-4. MDDC viability after BVDV infection. MDDC were infected with cpBVDV1b-TGAC, ncpBVDV1b-TGAN, ncpBVDV2a-28508-5 or ncpBVDV2a-1373 strain of BVDV. The viability of MDDC was measured by trypan blue exclusion assay and percentages of viable cells were calculated from 1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i.

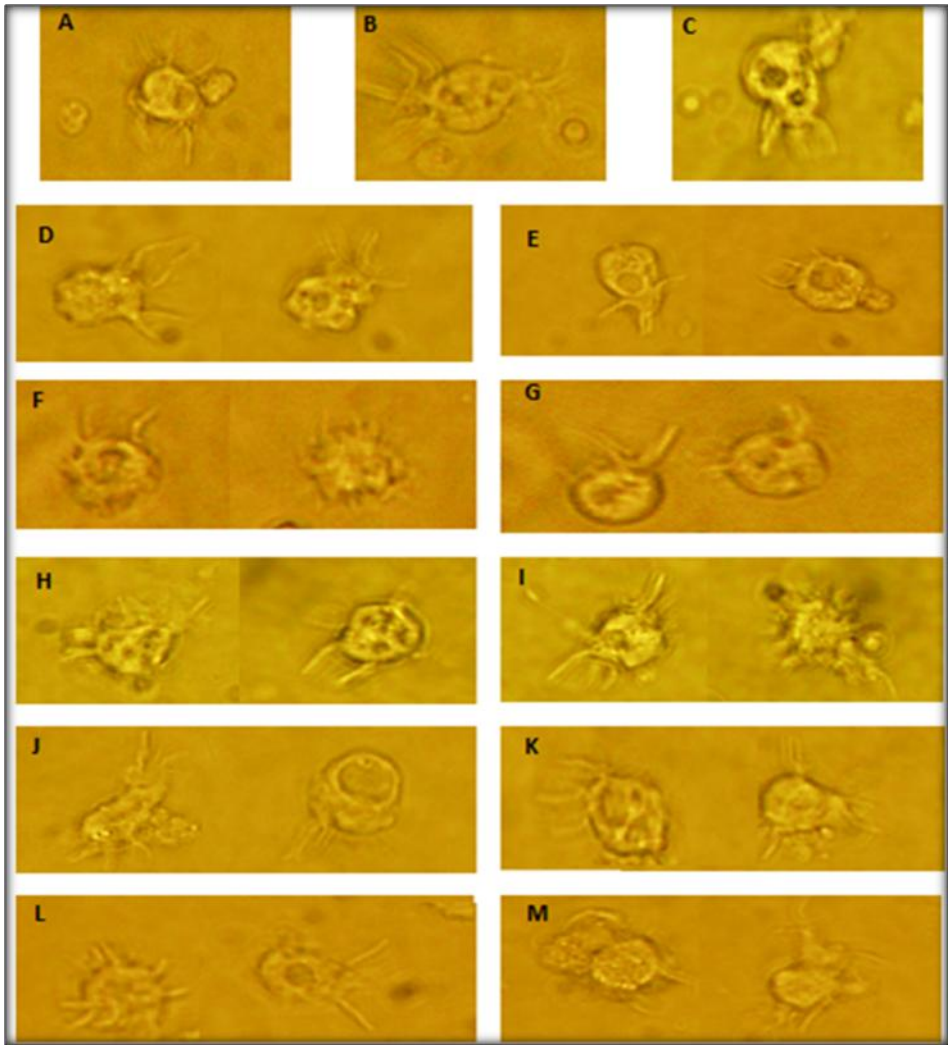

Figure 2-5. Effect of cp or ncp BVDV infection on MDDC. MDDC were infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV and examined for cytopathic effect at 1 hr, 6 hr, 12 hr or 24 hr p.i. A) mock-infected at 0 hr; B) mock-infected at 1 hr; C) mock-infected at 6 hr; D) mock-infected at 12 hr and E) mock-infected at 24hr; F) MDDC infected with cp BVDV1b-TGAC at 1 hr; H) MDDC infected with cp BVDV1b-TGAC at 6 hr; J) MDDC infected with cp BVDV1b-TGAC at 12hr; L)

MDDC infected with cp BVDV1b-TGAC At 24 hr; G) MDDC infected with ncp BVDV1b-TGAN at 1 hr; I) MDDC infected with ncp BVDV1b-TGAN at 6 hr; K) MDDC infected with cp BVDV1b-TGAN at 12hr; M) MDDC infected with cp BVDV1b-TGAN at 24 hr. It might make sense to add a label like TGAC 24 hr to each panel.

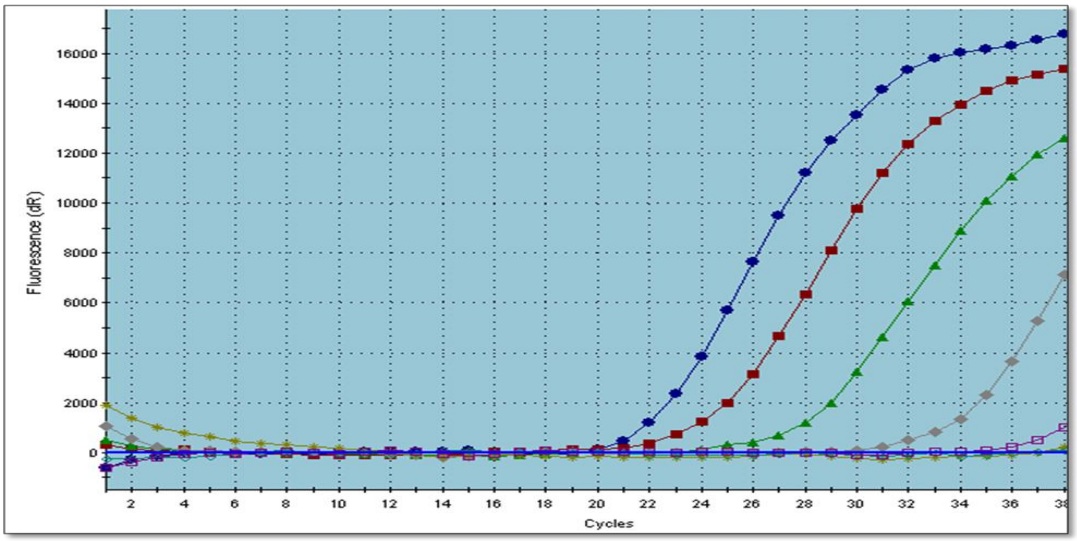

Figure 2-6. qR-TPCR amplification curve of serially diluted virus (spiked) of known concentrations. The MDBK cells were diluted in MEM medium to achieve final concentration 5x105 cells/ ml. The ncp BVDV2a-1373 strain of BVDV with known titer (7 log10/ml) was serially diluted as 1 in 10 in MDBK cells suspension. The viral RNA was extracted and quantified using qRTPCR.

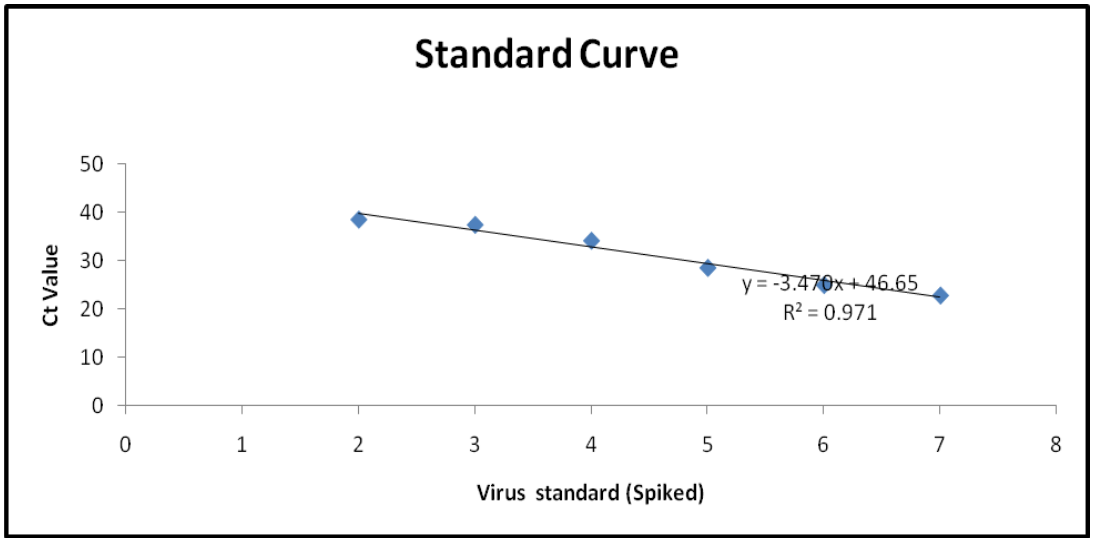

Figure 2-7. Standard curve with the known BVDV virus (spiked) concentrations.

The MDBK cells were diluted in MEM medium to achieve final concentration 5x105 cells/ ml. The ncp BVDV2a-1373 strain of BVDV with known titer (7 log10/ml) were serially diluted(spiked) as 1:10 in MDBK cells suspension. The viral RNA was extracted and quantified using qR-TPCR. The Ct (critical) values with known virus titers were used to plot the standard curve. The standard curve was used to calculate the approximate virus concentration (virus equivalence) in the samples.

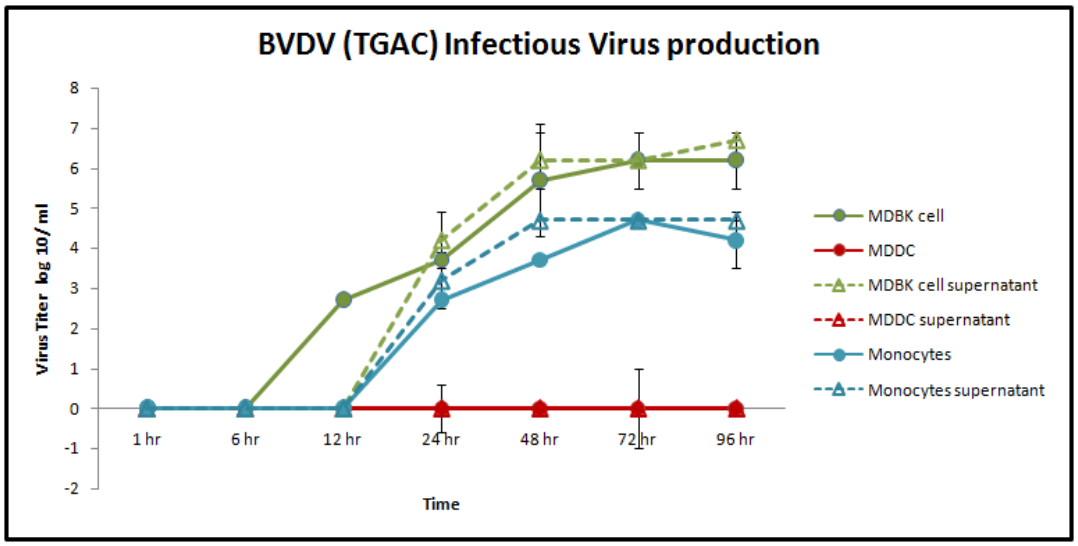

Figure 2-8. Virus production of cpBVDV1b-TGAC strain. The MDDC and MDBK cells were infected with 6 MOI of infection with cpBVDV1b-TGAC strain of BVDV. Cells and its supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr p.i. and analyzed for viral titer**.**

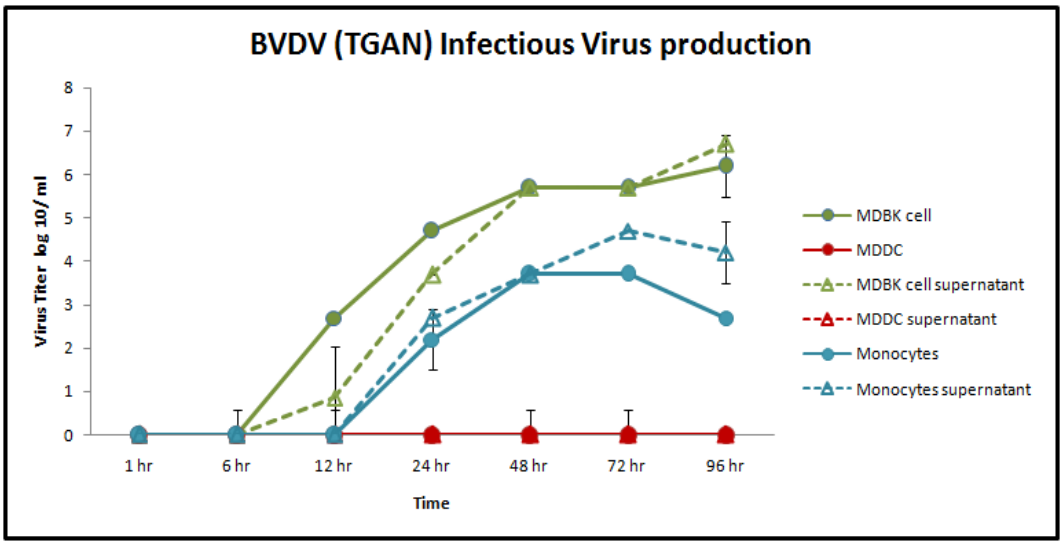

Figure 2-9. Virus production of ncpBVDV1b-TGAN strain of BVDV. The MDDC and MDBK cells were infected with 6 MOI of infection with ncp BVDV1b-TGAN strain of BVDV. Cells and its supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr p.i. and analyzed for viral titer**.**

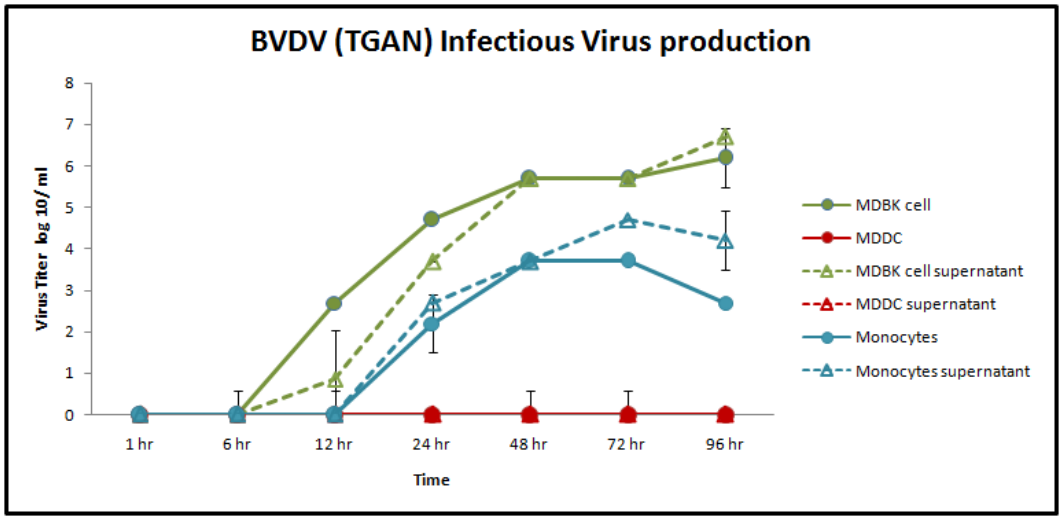

Figure 2-10. Virus production of ncpBVDV2a-28508-5 strain of BVDV. The MDDC and MDBK cells were infected with 6 MOI of infection with ncp BVDV2a-28508-5 strain of BVDV. Cells and its supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr p.i. and analyzed for viral titer**.**

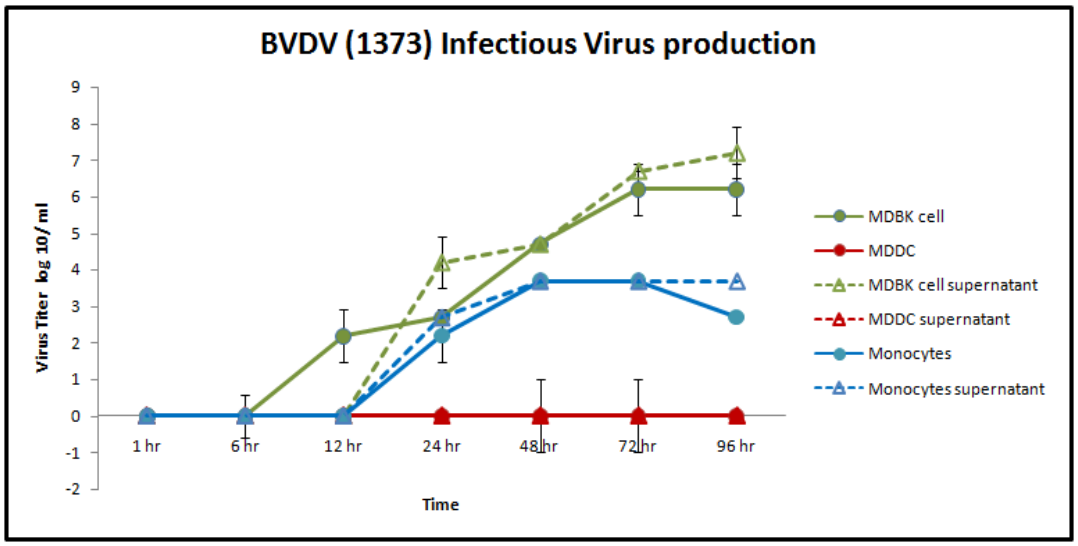

Figure 2-11. Virus production of ncpBVDV2a-1373 strain of BVDV. The MDDC and MDBK cells were infected with 6 MOI of infection with ncp BVDV2a-1373 strain of BVDV. Cells and its supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr p.i. and analyzed for viral titer.

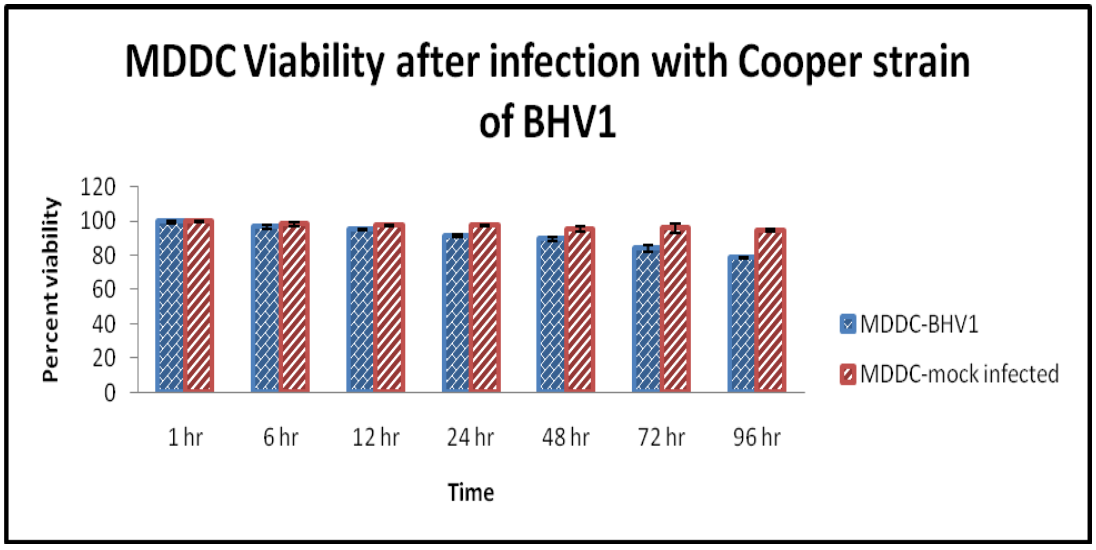

Figure 2-12. MDDC viability after BHV1 infection. The MDDC were infected with 6 MOI of infection with Cooper strain of BHV1. The cells were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr p.i. The viability of MDDC were examined through trypan blue exclusion assay.

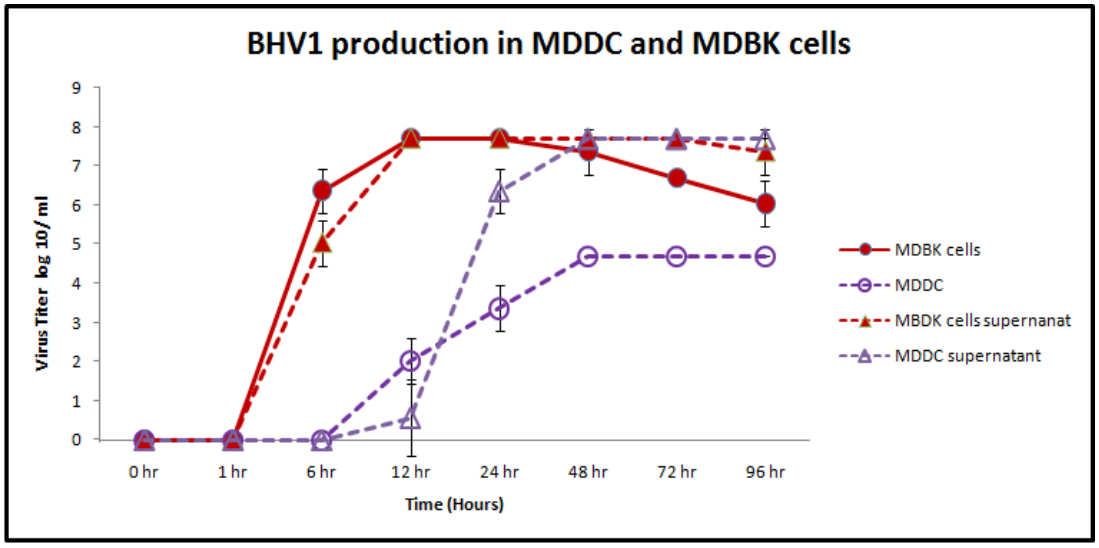

Figure 2-13. Virus production of BHV1. The MDDC or MDBK cells were infected with 6 MOI of infection with Cooper strain of BHV1. Cells and its supernatants were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr or 96 hr p.i. and analyzed for viral titer**.**

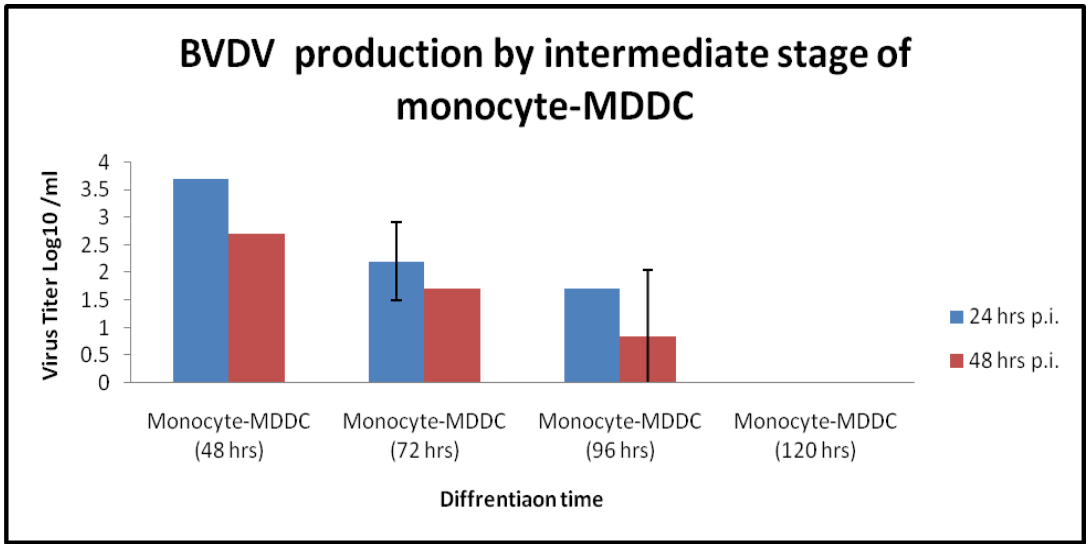

Figure 2-14. The virus production of ncp BVDV2a-1373 strain of BVDV by intermediate stage of MDDC. The intermediate stages of MDDC were harvested at 48 hr, 72 hr, 96 hr and 120 hr of differentiation. The intermediate stages of MDDC were infected with ncp BVDV2a-1373 strain of BVDV with 6 MOI of infection. The cells were collected at 24 hr and 48 hr p.i. and analyzed for viral titer.

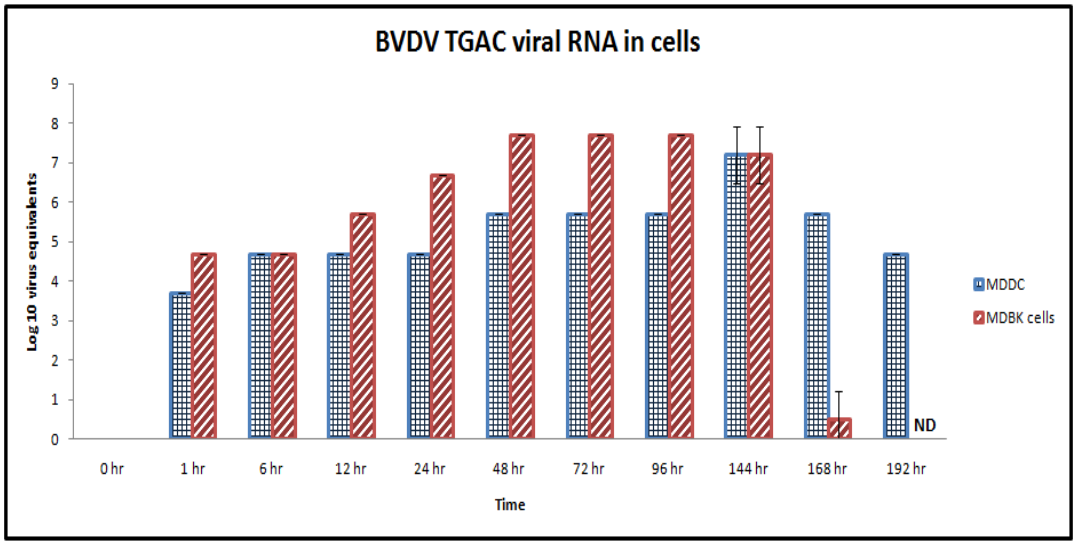

Figure 2-15. Replication of cpBVDV1b-TGAC viral RNA in MDDC or MDBK cells. MDDC or MDBK cells were infected with 6 MOI of infection with cpBVDV1b-TGAC strain of BVDV. Cells were collected at 0 hr, 1 hr, 6 hr, 12 hr, 72 hr, 96 hr, 144 hr or 168 hr p.i. The viral RNA was extracted from cells at each time point. Extracted RNA was quantified using qRTPCR at each time points.

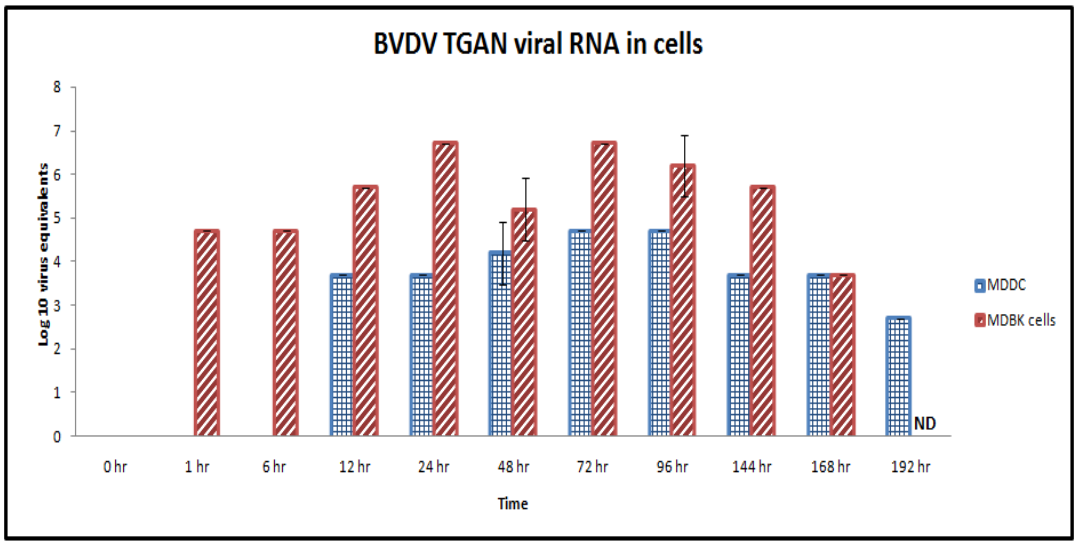

Figure 2-16. Replication of ncp BVDV1b-TGAN viral RNA in MDDC or MDBK cells. MDDC or MDBK cells were infected with 6 MOI of infection with ncp BVDV1b-TGAN strain of BVDV. Cells were collected at 0 hr, 1 hr, 6 hr, 12 hr, 72 hr, 96 hr, 144 hr or 168 hr p.i. The viral RNA was extracted from cells at each time point. Extracted RNA was quantified using qRTPCR at each time points.

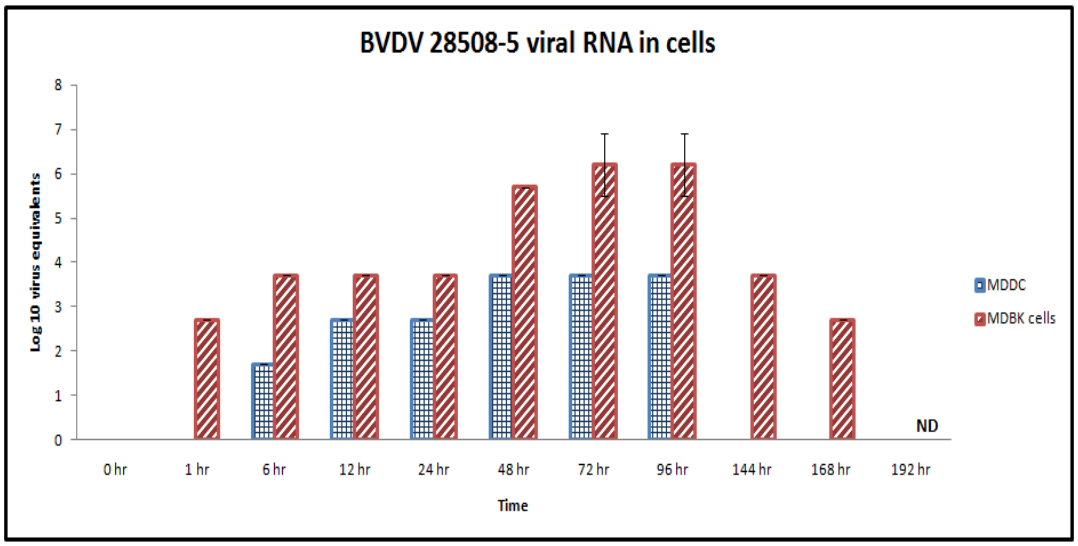

Figure 2-17. Replication of ncp BVDV2a-28508-5 viral RNA in MDDC or MDBK cells. MDDC or MDBK cells were infected with 6 MOI of infection with ncp BVDV2a-28508-5 strain of BVDV. Cells were collected at 0 hr, 1 hr, 6 hr, 12 hr, 72 hr, 96 hr, 144 hr or 168 hr p.i. The viral RNA was extracted from cells at each time point. Extracted RNA was quantified using qRTPCR at each time points.

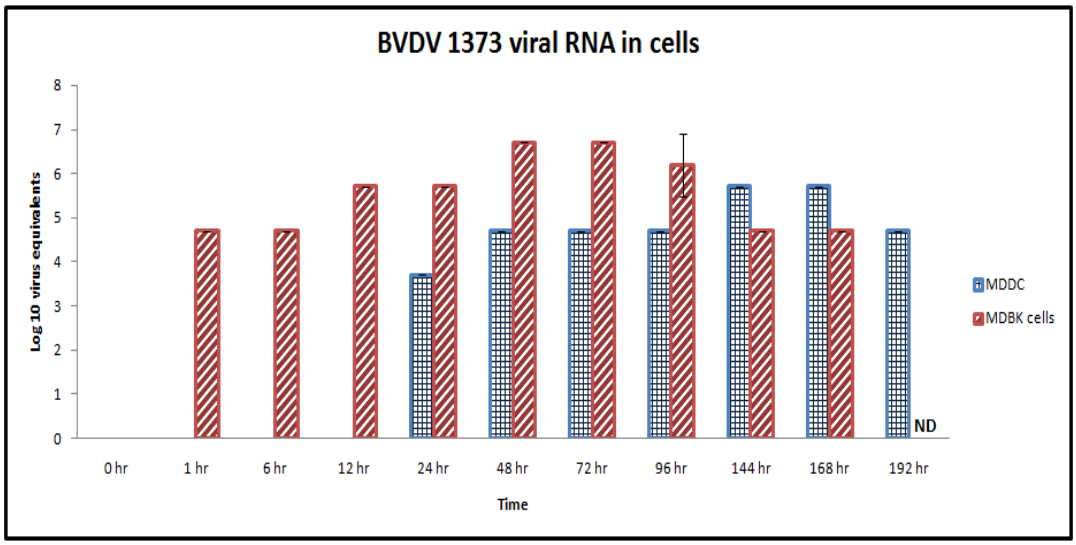

Figure 2-18. Replication of ncp BVDV2a-1373 viral RNA in MDDC or MDBK cells. MDDC or MDBK cells were infected with 6 MOI of infection with ncpBVDV2a-1373 strain of BVDV. Cells were collected at 0 hr, 1 hr, 6 hr, 12 hr, 72 hr, 96 hr, 144 hr or 168 hr p.i. The viral RNA was extracted from cells at each time point. Extracted RNA was quantified using qRTPCR at each time points.

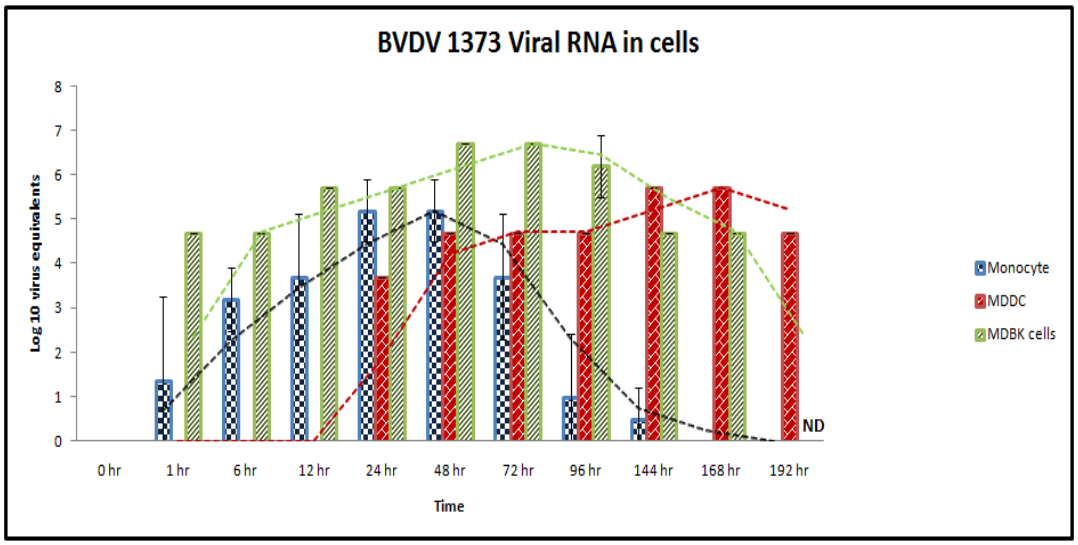

Figure 2-19. Replication of ncp BVDV2a-1373 viral RNA in monocytes, MDDC or MDBK cells. Monocytes, MDBK cells or MDDC were infected with ncp bvdv2a-1373 stain of BVDV at 6 MOI infection. Cells were collected at 0 hr, 1 hr, 6 hr, 12 hr, 72 hr, 96 hr, 144 hr or 168 hr p.i. Viral RNA was extracted from cells at each time point. Extracted RNA was quantified using qRT-PCR.

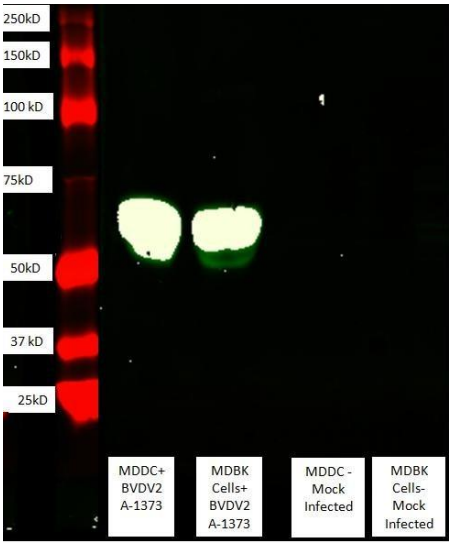

Figure 2-20. Presence of NS5A of BVDV in BVDV Infected MDDC through Western blot. The BVDV ncp BVDV2a-1373 infected MDDC cell lysate was separated using 12.5% SDS-PAGE resolving gel. Proteins were transferred to nitrocellulose membrane. The nitrocellulose membrane was stained for rabbit anti NS5A-BVDV antibody followed by goat anti-rabbit-IRDye 800CW. Images were obtained by using the Odyssey Imaging system and software (LI-COR Biosciences). Specific NS5A protein band was detected around 56 kD.

Viral Strain	Biotype	Genotype	Reference
BVDV1b-TGAC	CP	Type 1b	(Ridpath et al., 1991)
BVDV1b-TGAN	NCP	Type 1b	(Ridpath et al., 1991)
BVDV2a-1373	NCP	Type 2a	(Stoffregen et al., 2000)
BVDV2a-28508-5	NCP	Type 2a	(Liebler-Tenorio et al., 2003)

Table 2-1. BVDV Strains used in the studies

	Cells	Total number of cells from 60 ml heparinized blood	
		Brown Swiss	Holstein Friesian
1.	PBMC	$1.56\pm0.51 \times 10^8$	$5.36\pm0.73 \times 10^8$
2.	Monocytes	$1.36\pm0.40 \times 10^7$	$2.83\pm0.35 \times 10^7$
3.	MDDC	$4.2\pm0.72 \times 10^6$	$1.96\pm2.91 \times 10^6$

Table 2-2. The average yield of cells from 60 ml of heparinized blood.

Time	Control MDDC	MDDC infected with ncp BVDV2a-1373 strain of BVDV	MDDC infected with ncp BVDV2a-28508-5 strain of BVDV	MDDC infected with cp BVDV1b-TGAC strain of BVDV	MDDC infected with ncp BVDV1b-TGAN strain of BVDV
1 hr	99.33±1.15	100±00	99.33±1.15	99.33±1.15	98.66±1.15
6 hr	98.66±1.15	97.33±1.15	97.33±1.15	98.00±00	96.66±1.15
12 hr	97.33±1.15	96.66±1.15	96.00±2	96.66±1.15	95.33±2.309
24 hr	97.33±1.15	96.00±2.30	96.66±2.30	96.00±1.15	94.66±1.15
48 hr	96.66±1.15	96.00±3.4	96.66±2.30	94.00±3.46	94.66±1.15
72 hr	96.66±1.15	94.66±3.05	94.00±2	94.00±3.46	94.00±2.00

Table 2-3. MDDC viability after BVDV infection. MDDC were infected with either BVDV2a-1373, BVDV2a-28508-5, BVDV1b-TGAC or BVDV1b-TGAN strains of BVDV at 6 MOI of infection or mock infected control. MDDC were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. and examined for cell viability using trypan blue exclusion assay, the experiment was repeated three time and mean and standard deviation was calculated.

Time	BHV1-infected MDDC	Mock-infected MDDC
1 hr	99.33±0.57	99.66±0.58
6 hr	96.33±1.15	98.33±1.15
12 hr	94.66±0.58	97.33±0.58
24 hr	91.33±0.58	97.00±0.58
48 hr	89.33±1.52	95.00±1.52
72 hr	84.00±3.08	95.66±2.64
96 hr	78.37±0.58	94.33±0.58

Table 2-4. MDDC viability after BHV1 infection. The MDDC were infected with cooper strain of BHV1 with 6 MOI of infection. The percent viability of BHV1 infected MDDC was examined through trypan blue exclusion assay along with mock-infected MDDC.

CHAPTER 3.

THE EFFECT OF BVDV ON CELL SURFACE MARKERS OF BOVINE MONOCYTE-DERIVED DENDRITIC CELLS

ABSTRACT

Bovine viral diarrhea virus (BVDV) infections are a worldwide problem in cattle. One of the hallmarks of BVDV infections is immunosuppression. The exact mechanisms of this immunosuppression are not well characterized. In this chapter, the effect of BVDV infection on cell surface markers that are important for antigen presentation in dendritic cells (DC) were examined. Dendritic cells (DC) are a heterogeneous population of professional antigen presenting cells (APC) that are potent stimulators of naïve T-cells. DC activate both innate and adaptive immune responses by antigen presentation to T cells and by the production of pro- and anti-inflammatory cytokines. Bovine monocytes were cultured with bovine recombinant GM-CSF (100ng/ml) and IL-4 (200ng/ml). Over a period of 5-7 days in culture. The effect of BVDV infection on MDDC cell surface markers expression was measured. Four (4) BVDV strains were used including a noncytopathic (ncp) high virulence strain (BVDV2a-1373), a ncp typical virulence strain (28508-5), and a virus pair, cytopathic (cp)/(ncp) pair of viruses TGAN recovered from an animal that died of mucosal disease (BVDV1b-TGAC/BVDV1b-TGAN). The ncp BVDV strains reduced the MHCI and MHCII expression on MDDC cell surface. The maximum reduction in MHCI and MHCII was observed in the MDDC infected with the

high virulence strain (BVDV2a-1373) followed by typical virulence strain (ncp BVDV2a-28508-5) and BVDV1b-TGAN. BVDV1b-TGAN significantly reduced the MHCI as early as 1 hr p.i. All ncp strains used in the study significantly down regulated the CD86 expression at 48 hr p.i. The cp strain of BVDV up regulated the MHCI and MHCII expression in MDDC beginning at 1 hr p.i. while CD86 expression was significantly enhanced at 1 hr p.i. The down regulation of cell surface marker expression by ncp BVDV biotypes may be the one of the mechanism that functions in causes of immunosuppression and development of persistent infection. This enhancement of cell surface marker expression by cp BVDV may be one of the reasons for the strong immune response with these vaccines.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the most widespread cattle pathogens worldwide. BVDV infection in cattle herds result in major economic loss due to poor reproductive performance, immunosuppression and increased susceptibility to secondary infection. The BVDV strains can be divided into two biotypes, non-cytopathic (ncp) and cytopathic (cp) on the basis of their effect on cultured epithelial cells. Infection of bovine fetuses with ncp BVDV during first 40-120 days of pregnancy may result in persistently infected (PI) calves (**Chase et al., 2004).** The PI calves are immunotolerant and remain a source of infection to other animals. Superinfection of PI animals with

antigenically homologous cpBVDV strain results in a highly fatal form of BVDV associated disease known as mucosal disease (MD), **(Brownlie, 1990).**

Dendritic cells (DC) are antigen-presenting cells (APC) with a unique ability to induce immune responses by activating naïve T cells. The DC capture, process and present antigen to the T cells for adaptive immune response. DC not only activates T cells **(Steinman and Banchereau, 2007)** but they also induce immunological tolerance through anti-inflammatory cytokines **(Samarasinghe et al., 2006)** and generate regulatory T cells.. The activated T cells produce IL-2 (interleukin-2). IL-2 increases T-cell proliferation and prevents the induction of anergy and cell death **(Borthwick et al., 1996)**. The activated T helper-1 (Th1) cells either activate cytotoxic T cell to destroy infected cells or become T helper -2 (Th2) cells that facilitate B cell activation for antibody production **(Constant and Bottomly, 1997)**.

APC) including DC are target for many pathogens. A reproducible method to produce large number of monocyte-derived dendritic cells (MDDC) was used to study the interactions of pathogens with DC **(Mwangi et al., 2005; Werling et al., 1999).** Studies have shown that bovine MDDC are susceptible to various pathogens including bacteria such as *Salmonella typhimurium* **(Norimatsu et al., 2003)**, *Brucella abortus* **(Heller et al., 2012)**, *Mycobacterium paratuberculosis* **(Lei and Hostetter, 2007)** and viruses such as bovine respiratory syncytial virus **(Werling et al., 2002).** There have been two studies that have examined the ability of BVDV to infect MDDC **(Glew et al., 2003; Gibson et al., 2011)**. MDDC were susceptible to

BVDV infection with both ncp and cp strains isolated from a MD case **(Glew et al., 2003)** or from two different ncp strains that caused either severe acute or mild acute symptoms **(Gibson et al., 2011)**. Interestingly the cp strain of BVDV pair did not induce cytopathic effects on MDDC **(Glew et al., 2003)**.

Cell surface markers were affected following infection of MDDC with BVDV. The MDDC infected with ncp strain of BVDV up regulated MHCI expression while infection with cp BVDV reduced MHCI expression in MDDC. The MHCII expression was reduced in MDDC infected with either cp or ncp strains of BVDV while there was no effect on CD86 expression **(Glew et al., 2003).**

In this study, we evaluated the strain effect on cell surface markers expression on MDDC. The cells surface expression of MHCI, MHCII and CD86 on MDDC infected with a high virulence strain (BVDV2a-1373), a typical virulence strain (BVDV2a-28508-5) strains or cytopathic/noncytopathic pair of BVDV strains isolated from a MD case (BVDV1b-TGAC/BVDV1b-TGAN) was compared.

MATERIALS AND METHODS

Madin Darby bovine kidney (MDBK) cells

BVDV free MDBK cells (passage 95-110) were grown in minimal essential medium (MEM, Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% BVDV free fetal calf serum (FCS) (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 μg /ml) and used for viral propagation and titration.

Virus Strains and Preparation

The four BVDV strains were used in this study included a a pair of cp/ncp BVDV1b viruses, Tifton GeorgiA Cytopathic (BVDV1b-TGAC) and Tifton GeorgiA Non-cytopathic (BVDV1b-TGAN) recovered from an animal that died of mucosal disease **(Brownlie et al., 1984; Fritzemeier et al., 1995; Ridpath et al., 1991)**, a high virulence noncytopathic strain from the BVDV2 species (BVDV2a-1373) isolated following an outbreak of peracute BVDV in Canada **(Carman et al., 1998; Stoffregen et al., 2000)**; and 4) typical virulence noncytopathic virus from the BVDV2 species isolated from an asymptomatic persistently infected calf (BVDV2a-28508-5) **(Liebler-Tenorio et al., 2003)** (Table 2-1). Five (5) ml of $5x10^5$ MDBK cells/ml were seeded in T25 flasks and culture were grown to 60-70% confluency at the time of inoculation with virus. At the time of inoculation, the media was removed and 0.75 ml of virus inoculum with a multiplicity of infection [MOI] of one was added to each T25 flask. Virus was adsorbed for 1 hr at 37°C in a humidified CO2 incubator. After one (1) hr incubation, inoculum was removed and the cells were washed with sterile PBS. After washing, 5 ml of media was added to each flask. The cells were incubated at 37°C in a humidified CO2 incubator for 4-5 days for cultures infected with noncytopathic virus or or 70-80% cytopathic effect forcultures infected with cytopathic virus, TGAC. Cultures were harvested by two freeze thaw cycles. After 4-5 days for cultures infected with noncytopathic virus orof incubation cells were freezed at (-80˚C for 15 minutes followed by thawing at 25˚C).. The cell debris was pelleted by centrifugation at 1200g for 10 min at 4°C. The virus in the resulting

supernatants was titrated and the supernatants were, aliquoted and stored at -80 °C for further use.

The virus titration was determined by Reed and Munech method **(Reed and Munech, 1938**). Briefly MDBK cells were detached from tissue culture flask. The number of cells was adjusted to $5x10^5$ cells/ ml. One hundred eighty (180) µl cell suspensions was added to each well of 96-well plate. Twenty (20) µl of virus was added to the first row of the plate. The virus was mixed with MDBK cells and 20 µl of this dilution was added to next row to achieve 10 fold dilutions. No virus was added to the last two rows of wells. These wells served as negative controls. The four replicates of each dilution was performed to determine the virus titer. The plate was incubated at 37 °C at humidified incubator for next 4 days. The plate was examined every day for cytopathic effect (CPE) of the virus. The highest dilution showing CPE is used as end point to calculate the proportionate distance (PD). The PD was used to determine the viral concentration (TCID50) as per formula as described earlier (**Reed and Munech, 1938**).

5. Proportionate distance (PD) = (% CPE at dilution above 50%) – (50%)/ (% CPE at dilution above 50%)- (% CPE at dilution below 50%) (e.g. 60-50/60-0= 0.166)

6. Calculation of end point just next to 50% CPE and conversion into – Log (e.g.10^{-6} dilution would be -6)

7. Calculation of $TCID_{50.}$

8. $TCID_{50}$ for 20 µl= $^{(PD+ - Log\ dilution\ above\ 50\%)}$ (e.g. $1x10^{6.166}$)

For ncp BVDV, the same procedures with the exception of determining the end point since no CPE was produced. The end point for ncp BVDV was determined by staining the MDBK cells with anti-BVDV antibody (IDEXX Laboratories, Westbrook, ME, USA) followed by biotinylated rabbit anti-mouse IgG (Zymed, Invitrogen Corporation, Frederick, MD, USA) Steptavidin-HRP (Invitrogen Corporation, Camarillo, CA, USA) and AEC reagent (3 amino-9 ethyl-carbazole) (Sigma-Aldrich, St. Louis, MO, USA). The end point for ncp BVDV was determined by the presence of red stained cell showing BVDV protein.

Animals

Six (6) Brown Swiss female calves (8-12 months of age) housed at Dairy Farm, South Dakota State University (SDSU), Brookings, SD, USA were used in this study. All animals were healthy. The SDSU Institutional Animal Care and Use Committee approved animal handling and blood collection.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated as per method previously described **(Ulmer et al., 1984)** and differentiated into MDDC **(Mwangi et al., 2005)** with following modifications; 2-mercaptoethanol, 2 mM GlutaMax and 25 mM HEPES were replaced with 1mM sodium pyruvate (personal communication, Dr Waithaka Mwangi, Texas A&M University, USA) and the medium used for MDDC differentiation was RPMI-1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100μg/ml), bovine recombinant

granulocyte macrophage-colony stimulating factor (GM-CSF (100ng/ml) and IL-4 (200ng/ml). In the previous method fresh, complete RPMI-1640 medium and cytokines was added at every 3rd day while in current study 750 µl fresh, complete RPMI-1640 medium with cytokines was added every 2nd day (every other day). The addition of sodium pyruvate and increasing the concentration of FBS from 10% to 20% improved the differentiation of MDDC. The beneficial effect of sodium pyruvate may be due to its protective effect to nutrients in media and providing energy to the cell **(Giandomenico et al., 1997).**

Briefly, sixty (60) ml of heparinized venous blood was collected from healthy calves. The buffy coat layers were separated by centrifuging the blood at 1100g for 30 minutes at 4°C. The cells from buffy coat layer were suspended into heparinized (10 U/ml) PBS in 1:3 ratio. The diluted cells were overlaid on 3ml, 65% Percoll (GE Healthcare Biosciences**,** Pittsburgh, PA, USA) in 15 ml conical tubes (Falcon, Oxnard, CA, USA) and centrifuged for 30 min, at 1100g at 4°C. The white cell layer of PBMC was aspirated by pipette from the interphase. The collected PBMC were suspended in heparinized PBS (10 U/ml) and pelleted by centrifugation at 1700 rpm for 15 min at 4°C. The PBMC were washed two times by suspending them in PBS and centrifugation at 1700 rpm for 15 min at 4°C. Finally, PBMC were suspended in RPMI 1640 medium supplemented with 10% FBS, 1mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100µg/ml) to achieve final concentration of 1x 10^7cells/ ml. Three (3) ml

of the cell suspension was added to each well of a 6-well plate (Falcon, Oxnard, CA, USA) and incubated at 37°C in a humidified CO_2 incubator for 3 hr.

Separation of adherent monocytes

The monocytes were isolated by the plastic adhesion method **(Mwangi et al., 2005)**. The PBMC were cultured in 6-well plates for 3 hr at 37°C in a humidified CO_2 incubator. The unattached cells were discarded and plates were washed 4 times with PBS. The attached cells were detached with Accuatase (eBioscience, San Diego, CA, USA). Detached cells were washed two times by suspending them in PBS and centrifugation at 500g for 15 min at 4°C.

Monocyte-derived dendritic cell (MDDC) culture

The accuatase-detached cells were characterized as $MHCI^+$ $MHCII^+$ and $CD14^+$ monocytes based on flow cytometer analysis done on a FACScan (Becton-Dickson, Mountain View, CA) using MHC-I (H58A), MHC-II (H42A), CD21 (BAQ15A) andCD14 (MM61A) primary antibodies (VMRD Inc., Pullman, WA, USA). The harvested CD14+ monocytes were counted and the yield determined. The monocytes were differentiated into monocyte-derived dendritic cells (MDDC) **(Mwangi et al., 2005)** with some modifications as described above. Briefly, the monocytes were diluted in RPMI 1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), bovine recombinant GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) kindly provided by Dr. Waithaka Mwangi (Texas A&M University, USA) to achieve the final concentration of $5x10^5$cells/ml. Three (3) ml of this cell suspension

was added to each well in 6-well plates. The cells were incubated at 37°C in a humidified CO_2 incubator for 7 days. Seven hundred and fifty µl (750 µl) of RPMI 1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100µg/ml), bovine recombinant GM-CSF (100 ng/ml) and IL-4 (200 ng/ml) was added to each well at alternate day. At day seven, differentiated MDDC were collected.

Virus infection of MDDC

After 7 days of culture, MDDC were collected by aspiration and centrifuged at 500g at 4°C for 15 min in 15 ml conical tubes. MDDC were then suspended in RPMI 1640 medium supplemented with 20% FBS, 1mM sodium pyruvate, penicillin (100 U /ml) and streptomycin (100 µg /ml), bovine recombinant GM-CSF (100ng/ml) and IL-4 (200ng/ml) to final concentration $5x10^5$ /ml. One (1) ml of the cell suspension was added to each well of the 24-well plates. An aliquate of BVDV inoculum with MOI of 6 was added to each well. Mock-infected MDDC with no virus were used as control. BVDV and mock-infected MDDC were collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. from different wells to measure their viability and cell surface marker expression. At least three replicates were performed to determine the final result. These time points were chosen because most cell surface marker genes are affected within 24 hr of infection in human monocyte-derived macrophages (MDM) or MDDC **(Lehtonen et al., 2007)** and maximum BVDV titer was observed at 72 hr post infection in BVDV permissive cells (e.g. MDBK cells) in previous studies in our laboratory.

Change in cell surface marker expression following BVDV infection

The mock-infected and BVDV –infected MDDC were collected and stained for MHCI, MHCII or CD86 at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. For staining cells number was adjusted to $1x10^6$/ml. Three primary mouse mAb antibodies for MHC-I (H58A), MHC-II (H42A) and CD86 (IL-A190A) (MM61A)(VMRD Inc., Pullman, WA, USA) were used. The primary antibodies were diluted 1:100 in PBS containing 1% FBS. The 100 µl of cells suspension was incubated with 50µl of diluted primary antibodies at 4°C for 10 min followed by washing by centrifugation at 200g for 4 minutes at 4 °C in round bottom 96 well plates. After centrifugation cell pallet was suspending them in 200 µl PBS. After primary staining and washing, cells were incubated with 50 µl FITC labeled anti-mouse secondary antibody (VMRD Inc., Pullman, WA, USA) which was diluted 1:1000 dilution in PBS containing 1% FBS at 4°C for 10 min. After incubation with secondary antibody, cells were washed two times with PBS. Cells were suspended for in 200 µl of 1% paraformaldehyde for fixing. The mean fluorescent intensity (MFI) in fixed cells was analyzed using FACSCalibur software (BD Biosciences). At least 20,000 cells were run at each time points and each experiment was repeated at least 3 times.

Statistical analysis

Three replicates were performed to confirm the reproducibility of the method to differentiate the monocyte to MDDC. The percent change in cell surface marker expression in BVDV-infected MDDC were done three times in different trials. The

significance difference in cell surface markers from its time point control was calculated by paired t-test ($p<0.05$) **(Glantz, 2002).**

RESULTS

Effect of BVDV on MHC I, MHC II and CD86 expression

The MDDC were infected with ncp BVDV2a-1373, BVDV2a-28508-5, BVDV1b-TGAN strains or cp BVDV1b-TGAC strain of BVDV at a MOI of 6. The MDDC were stained for MHCI, MHCII or CD86 and fixed at 0 hr, 1 hr, 6 hr , 12 hr p.i. 24 hr, 48 hr or 72 hrs p.i. to measure the changes on cell surface expression following BVDV infection. The experiments were repeated at least three times using three different animals. The mean MFI in mock-infected MDDC at zero time point was treated as 100%. The mock-infected MDDC at each time point were used as control. The percent change at each time was calculated.

MHCI

The MFI of MHC I in MDDC infected with cp BVDV1b-TGAC increased 12 hr p.i. The MFI of MHC I in MDDC infected with cp BVDV1b-TGAC strain of BVDV increased around 33% (133.50±11.88%), 89% (189.10±34.3%) and 130% (230.2±5.22%) at 1 hr, 6 hr and 12 hr p.i. respectively, compared to mock-infected MDDC (Table 3-1, Figure 3-1). Similarly, the MFI of MHC I also increased from 24 hr p.i. to 72 hr p.i. following TGAC infection .The MFI of MHCI increased around 12% (112.22±17.29%), 44%(144.60±53.14%) and 58% (158.16±52.43%) at 24 hr, 48hr and 72 hr p.i. as compared to mock-infected MDDC respectively (Table 3-1, Figure 3-1). Small changes

in MHCI expression was observed at (98.32±1.38%) at 1 hr; (99.24±1.09%) at 6 hr,(100.63±1.2%) at 12 hr, (91.44±5.44%) at 24 hr, (90.47±5,85%) at 48 hr and (78.90±25.81%) at 72 hr in time-matched mock-infected MDDC (Table 3-1, Figure 3-1). The MHC I expression on BVDV1b-TGAC infected MDDC was significantly up regulated following 1 hr, 6 hr, 12 hr, 24 hr and 48 hr p.i. of TGAC as compared to controls ($p<0.05$).

In contrast, the MFI of MHC I decreased at over time in MDDC infected with the three different ncp BVDV strains. The MFI of MHCI in ncpBVDV1b- ncpBVDV1b-TGAN infected MDDC decreased around 18% (82.15±3.72%), 30% (70.58±0.76) and 46% (54.61±2.03) at 1 hr, 6 hr and 12 hr p.i. respectively and 23%(77.96 ±9.93%), 35% (65.23 ± 11.90%) and 36% (64.15± 9.25%) at 24hr, 48hr and 72 hr p.i respectively (Table 3-1, Figure 3-1, Figure 3-5). The down regulation of MHCI expression following ncpBVDV1b-TGAN infection was significant different at 1 hr, 6 hr, 12 hr and 24 hr p.i. as compare to mock-infected MDDC ($p<0.05$). The MFI of MHCI in MDDC infected with ncpBVDV2a-28508-5 was reduced 22% (78.62±17.19%), 36%(64.05±37.01%) and 43% (57.94±27.84%) at 1 hr, 6 hr, and 12 hr respectively and 32% (68.14±18.62%), 57% (43.57±6.63%) and 59% (41.30±5.95%) at 24 hr, 48 hr and 72 hr p.i. respectively as compared to mock-infected MDDC (Table 3-1, Figure 3-2, Figure 3-7). The down regulation of MHCI in ncpBVDV2a-28508-5 infected MDDC was significantly lower at 24 hr and 72 hr p.i. ($p<0.05$). The MFI of MHCI in MDDC infected with ncpBVDV2a-1373 strain of BVDV reduced expression around 14% (86.73±18.33%), 40%

(60.26±18.02%) and 48%(52.31±20.05%) at 1 hr, 6 hr and 12 hr p.i. respectively and 35% (64.32±7.41%), 59% (41.85±13.64%), and 60% (40.10±7.18%) at 24 hr, 48 hr and 72 hr p.i. respectively as compared to mock-infected controls (Table 3-1, Figure 3-2, Figure 3-6). BVDV2a-1373 infection to MDDC significantly down regulated the MHCI expression at 24 hr and 48 hr p.i. as compare to time matched control ($p<0.05$).

MHCII

There was also a biotype difference in MHCII expression. The cp biotype cpBVDV1b-TGAC unregulated the MHCII expression while its homologue ncpBVDV1b-TGAN strain down regulated the MHCII expression. cpBVDV1b-TGAC increased the MHCII expression during the course of infection. The MHCII expression increased around 19% (119.15±9.23%), 23% (123.96±12.17%) and 50% (150.49±24.69 %) at 1 hr, 6 hr or 12 hr p.i. respectively and ~22% (122.19±31.38%), ~40% (140.97±48.37) and ~56% (156.83±54.48%) at 24 hr, 48 hr and 72 hr p.i. respectively in cpBVDV1b-TGAC-infected MDDC as compared to mock-infected controls (Table 3-2, Figure 3-2, Figure 3-4). In contrast to its cp homologue, the ncpBVDV1b-TGAN down regulated that MHC II expression. The MFI of MHC II ncpBVDV1b-TGAN- infected MDDC was reduced ~10% (90.02±0.101%), ~28% (76.62±3.09%) and ~33% (67.32±.006%) at 1 hr, 6 hr and 12 hr p.i. respectively and ~2% (98.27±0.72%) at 24 hr p.i., ~21% (79.78± 23.69%) at 48 hr p.i. and ~21% (79.33±19.33%) at 72 hr p.i. as compared to mock-infected control (Table 3-2, Figure 3-2, Figure 3-5). The down

regulation of MHCII expression in ncpBVDV1b-TGAN infected MDDC was significantly different than the mock infected controls at 6 hr and 12 hr p.i. ($p<0.05$).

The MHCII expression also was reduced in MDDC infected with ncpBVDV2a-28508-5 strain of BVDV. The ncpBVDV2a-28508-5 infection in MDDC reduced MHCII expression ~9% (89.90±20.73%), 33 % (67.67±8.06%) and 36% (64.11±8.85%) at 1 hr, 6 hr and 12 hr p.i. respectively and ~22% reduction (78.10±0.38%) at 24 hr;~39% reduction (61.41±14.62%) at 48 hr and ~44% reduction (56.44±13.01%) at 72 hr p.i. (Table 3-2, Figure 3-2, Figure 3-7). The down regulation of MHCII on MDDC by mild acute 28508-5 was significantly different than its time point control at 6 hr, 12 hr and 72 hr p.i. ($p<0.05$).

Infection of MDCC with the high virulent ncpBVDV2a-1373 strain of BVDV reduced MHCII expression during the course of infection [~9%(89.04±15.59%) at 1 hr, 37% (63.81±1.57%) at 6 hr and 38% (62.79±5.10%) at 12 hr and ~29% (81.15±10.71%) at 24 hr, ~38% (63.65±8.03%) at 48 hr and 45% (55.10±8.39%) at 72 hr p.i]. (Table 3-2, Figure 3-2, Figure 3-7). The ncpBVDV2a-1373 strain of BVDV significantly down regulated the MHCII expression at 12 hr and 72 hr p.i. as compared to mock-infected controls ($p<0.05$).

CD86

Like MHCII expression, the BVDV biotype had affected the CD86 expression. The cpBVDV1b- TGAC strain of BVDV increased CD86 expression ~11% (111.40±3.55%), 5% (105.82±0.29%) and 7% (107.44±5.08%) at 1 hr, 6 hr and 12 hr p.i.

respectively. The cpBVDV1b-TGAC did not affect CD86 MFI at 24 hr p.i. (92.83±23.47%) but dramatically increased MFI by ~150% at 48 hr [147% (247.498±172.29%)] and 161% (261.22±162.00) 72 hr p.i. (Table 3-3, Figure 3-3, Figure 3-4). Statistically, cpBVDV1b-TGAC significantly up regulated the CD86 expression at 1 hr p.i.

This was in contrast to ncpBVDV1b-TGAN strains that decreased expression of CD86 by 1% (99.48±6.13%), 5% (95.09±0.29%) and 3% (93.55±2.49%) 1 hr, 6 hr and 12 hr p.i. respectively and ~ 20-30% from 24 hr to 72 hr p.i. [~20% (80.73±2.65%), ~29% (71.26±2.21%) and ~28% (72.64±1.38%) at 24 hr, 48 hr and 72 hr p.i. (Table 3-3, Figure 3-3, Figure 3-5). The ncp BVDV strains including high virulent ncpBVDV2a-1373, typical virulent ncpBVDV2a-28508-5 and ncpBVDV1b-TGAN significantly reduced the CD86 at 48 hr p.i. ($p<0.05$).

The CD86 expression in MDDC infected with ncpBVDV2a-1373 increased around 8% (108.42±11.49%), 11% (111.14±17.02%) and 6% (106.47±9.3) at 1 hr, 6 hr and 12 hr p.i. than reduced ~23% (77.75±10.55%), ~28% (72.16±4.63%), and ~29% (71.36±4.08%) at 24 hr, 48 hr and 72hr p.i. respectively (Table 3-3, Figure 3-3, Figure 3-6).

The ncpBVDV2a-28508-5 strain of BVDV reduced CD86 expression ~ 1% (99±10.08%),~ 2% (98.17±8.96%) at 1 hr and 6 hr p.i. followed by a slight upregulation at 12 hr p.i. as ~6% (106.22±20) while down regulated CD86 expression at 24 hr, 48 hr

and 72 hr p.i. as ~ 21% (79.61±13.77%) , ~24% (76.53±10.84%) and~ 24% (76.31±11.03%) respectively (Table 3-3, Figure 3-3, Figure 3-7).

DISCUSSION

There were seveal key findings on the effect of BVDV on antigen presentation and co-stimulation marker expression/concentration). The cp BVDV1b-TGAC strain of BVDV enhanced the MHCI, MHCII and CD86 concentration during the course of infection. The cp BVDV1b-TGAC strain of BVDV significantly up regulated the MHCI expression at 1 hr, 6 hr, 12 hr, 24 hr and 48 hr p.i. The cp BVDV1b-TGAC also significantly enhanced the CD86 expression at 1 hr p.i. as compared to its time point control ($p< 0.05$) and enhanced expression at 48 and 72 hrs. The ncp strains of BVDV including the high virulent ncp BVDV2a-1373, typical virulent ncp BVDV2a-28508-5 and ncp BVDV1b TGAN all reduced the MHCI, MHCII and CD86 expression during the course of infection. The ncp BVDV1b-TGAN strain of BVDV significantly down regulated MHCI expression starting from 1 hr p.i. to 12 hr p.i. while high virulent ncp BVDV2a 1373 down regulated the MHCI expression at 24 hr and 48 hr p.i. and typical virulent ncp BVDV2a-28508-5 down regulated the MHCI expression at 24 hr and 72 hr p.i. ($p< 0.05$).

Ncp BVDV1b-TGAN infection significantly down regulated the MHCII expression at 6 hr and 12 hr p.i. while typical virulent ncp BVDV2a-28508-5 significantly down regulated the MHCII expression at 6 hr, 12 hr and 72 hr p.i. and high virulent ncp BVDV2a-1373 significantly down regulated the MHCII expression at 12 hr

and 72 hr p.i. All ncp BVDV strain used in the study significantly down regulated the CD86 expression at 48 hr p.i. ($p < 0.05$). In another study with bovine MDM showed the upregulation of MHCI in MDM following infection with cp BVDV while down regulation of MHCI expression in MDM infected with ncp strain of BVDV. Whereas both cp and ncp strains down regulated the MHCII expression in MDM **(Chase et al., 2004).** The diffrence with our result may be due to diffrence in the isolation and culturing method. The MDDC were non attached, CD14- cells while MDM used in that study were adherent CD14+ cells.

One *in vivo* experiment, examined the effect of infection of calves with the type 2 ncp Canadian 24515 field isolate of BVDV on expression of MHCII and CD86 on PBMC. The percentages of B7 (CD80/86) and MHCII expressing peripheral blood mononuclear cells (PBMCs) were not changed in BVDV infected calves as compared to mock-infected calves **(Archambault et al., 2000).** In another *in vivo* experiment, analysis of lymph node and Peyer's patch cells from BVDV-infected calves found a 30% to 50% decrease in MHC II-expressing cells **(Brodersen and Kelling, 1999).** These results indicates that BVDV does not have effect on (CD80/86) and MHCII expression PBMCs while these cell surface marker (MHCII) is affected in when cell matured/differentiated in secondary lymphoid organs.

In another study, a ncp strain effect was demonstrated on MHCII expression in 1-day-old gnotobiotic calves. The calves were exposed to one of 2 noncytopathic isolates of BVDV (ncp-BVDV 7937 and ncp-BVDV 126). Phenotypic analyses of peripheral

blood mononuclear leukocytes was done at 3, 7, and 10 days p.i. The percentage of MHCII positive cells from calves exposed to ncp-BVDV 7937 was significantly higher than calves exposed to ncp-BVDV 126 on day 3 and 10 p.i. On day 10 p.i., calves exposed to ncp-BVDV 7937 also had a significantly higher number of B cell than calves exposed to ncp-BVDV 126 and non exposed control calves. Calves exposed to ncp-BVDV 126 had a significantly higher percentage of CD2 (BoCD2) positive T cells than calves not exposed to BVDV, indicating that ncp-BVDV 7937 up regulated the MHCII expression that further facilitate the B cell proliferation **(Marshall et al., 1994).** The difference from current finding where ncp strain of BVDV down regulated the MHCII may be due to difference in experimental design. The above experiment was carried out *in vivo* while current study was done in *in vitro* with different strain of viruses.

What is the mechanism responsible for these changes in MHCI and MHCII expression? Changes in surface marker expression could be due to differences in interferon expression between cp and ncp BVDV in MDDC. Type 1 IFN plays an important role in upregulation of MHCI expression **(Tovey et al., 1996)**. Reduced type 1 IFN production in ncp BVDV infected cells maybe a reason for the down regulation of MHCI and MHCII in ncp-infected MDDC while upregulation of MHCI and MHCII in cp BVDV infected MDDC may be due to an increase in type 1 interferon **(Charleston et al., 2001).** A study has shown that the BVDV Erns and Npro viral proteins inhibit the IFN production. The BVDV Erns protein inhibits IFN expression induced by extracellular viral RNA while BVDV Npro protein promotes the degradation of the transcription factor

IRF-3, which effectively blocks IFN expression in BVDV-infected cells **(Peterhans and Schweizer, 2013).**

Another possible mechanism may be a direct effect on MHC expression. Hepatitis C Virus (HCV), another virus of Flaviviridae family, changed MHCI expression in infected cultured cancerous cells lines (human hepatoma and mouse lymphoma cells) **(Mullbacher and Lobigs, 1995; Herzer, Falk et al., 2003; Tardif and Siddiqui, 2003)**. The reduced MHCI expression in HCV infected cells was due to disruption in MHCI protein folding and assembly. HCV replicons induce endoplasmic reticulum (ER) stress, which results from a decline in protein glycosylation. Decreasing protein glycosylation disrupts protein folding and preventing the assembly of MHC class I molecules and expression **(Tardif and Siddiqui, 2003)**. Similarly, HIV **(**Human immunodeficiency virus) reduces MHCI and CD86 expression via its Nef protein in 293T cell line (human embryonic kidney cell line), human monocytic U937 cell line as well as in mouse macrophages and dendritic cells **(Piguet et al., 2000; Chaudhry et al., 2005).** The Nef protein binds and endocytose the MHC class I molecules by the ARF6 pathway and resulting in reduced MHCI expression **(Piguet et al., 2000)**. The mechanism by which BVDV alter the cell surface marker expression need to be explored for better understating immunosuppression caused by BVDV.

Differential detergent fractionation (DDF) analysis of bovine monocytes showed that fifty-three (53) bovine proteins involved in immune function of professional APC, which were altered following BVDV infection. These molecules include adhesion

molecules, toll-like receptors (TLR1, 6 and 8), antigen uptake and MHC class I- and II, cytokines, and growth factors synthesis **(Lee et al., 2006)**. These results indicated that BVDV affected the immune response via both innate as well as adaptive immune systems components. A previous study was done to determine the effect of noncytopathic (ncp) and cytopathic (cp) BVDV on gene expression of TLR, type I IFN, pro-inflammatory and Th1/Th2 cytokines. The NADL strain of BVDV1a was used as cp biotype while New York 1 (NY-1) of BVDV1b was used as ncp biotype. **(Lee et al., 2008).** The results of this study indicated that the ncp strain of BVDV (NY1) up regulate the TLR3 and Type I IFN that is different than other finding where they found that ncp BVDV do not induce the type 1 IFN *in vitro* **(Schweizer and Peterhans, 2001)**. Our finding concur with those of **(Schweizer and Peterhans, 2001)** demonstrating that ncp BVDV do not induce type 1 IFN by down regulating MHCI and MHCII expression on MDDC infected with ncp strains of BVDV. The above experiment **(Schweizer and Peterhans, 2001)** has one limitation as they did not took a cp and ncp BVDV pair to see the difference in both the biotypes and virus taken are from two different subgenogroup of BVDV1. In another previous study, the proteins related to antigen pattern recognition, uptake and presentation to immunocompetent lymphocytes were analyzed in cp NADL BVDV infected monocytes through differential detergent fractionation (DDF) analysis. Eighteen (18) MHC proteins (4%) out of 445 were significantly altered in cp NADL BVDV infected bovine monocytes. Nine (9) MHC class I and six (6) MHC class II proteins, including the DQ isotype were significantly down-regulated in cp BVDV infected bovine

monocytes while MHC class II DR-beta chain protein was increased in cp BVDV infected monocytes **(Lee et al., 2009)**. These findings also support that BVDV infecting affect the cell surface marker expression in infected cell. Our finding showed the up regulation of MHCI and MHCII expression on MDDC. The differences with above study may be due to difference in cell types (monocytes) and technique used (DDF). The above study has one limitation that the DDF indicates the amount of proteins translated in the cell while it does not show actual protein expression on cell surface. The alteration in cellular proteins following BVDV infection may be associated with changes in cell surface expression. MHC class II molecules are physically and functionally associated with TLR2 in lipid raft domains of the cell membrane **(Frei et al., 2010)**. Previously it has been reported that monocyte-derived macrophages (MDM) infected with ncp BVDV-28508-5, significantly ($p<0.05$) decreased the amount of mRNA of TLR-2 **(Tigabu et al., 2004)**. The reduction in MHCII expression in ncp BVDV-28508-5 infected cell may be due to low expression of TLR-2.

We observed down regulation of CD86 expression following BVDV infection with ncpBVDV but up regulation following cpBVDV infection. Increased expression of CD86 may be due to effect of type one IFN. It has been shown that injecting IFN alpha/beta in mice increased expression of CD86 in murine DC **(Montoya et al., 2002)**. Similarly addition of IFN-alpha to the human PBMC culture greatly enhanced CD86 concentrations on the surface of developing dendritic cells **(Radvanyi et al., 1999).** It has been obseved that there are reduced level of co-stimulatory molecules CD80 and CD86

and pro-inflammatory cytokines TNF-alpha, IL-1beta, and IL-6 expression in bovine monocytes after infection with either cp or ncp strain of BVDV **(Lee et al., 2008).** Another study conducted with MDDC found no effect of cp BVDV infection on CD86 expression while ncp BVDV increase CD86 expression **(Glew et al., 2003)**. Difference in results may due to the different monocyte isolation and MDDC culturing methods. In the Glew study, the MDDC were $CD14^{+}$ while in the current study the MDDC were determined as non-adherent $CD14^{-}$cells **(Glew et al., 2003; Werling et al., 1999)**. The presence of CD14 indicates a different cell population with different functions. The CD14 has been shown to function as a receptor for a complex of LPS and LPS binding protein **(Haziot et al., 1993)**. Generally CD14 is expressed more in adherent cells like macrophage and monocytes. The CD14 expressing cells have major role in production of pro-inflammatory cytokines **(Ebong et al., 2001)** and recruitment of neutrophils **(McAvoy et al., 2011)** while DC maintain the immune balance by its pro-inflammatory and anti-inflammatory cytokines and induction of tolerance.

In brief, the results of this study suggested that Brown Swiss breed of cattle is more efficient for *in vitro* differentiation of MDDC then Holstein Friesian. The ncp strain of BVDV reduced MHCI and MHCII expression while cp strain enhances the MHCI, MHCII and CD86 expression on MDDC. The modified live BVDV vaccines contain cp strains of BVDV. The encouraging result of modified live BVDV may be to due to up regulation of the cell surface marker expression specially CD86 following vaccination. Further studies need to be done with other cp BVDV strains to confirm this finding.

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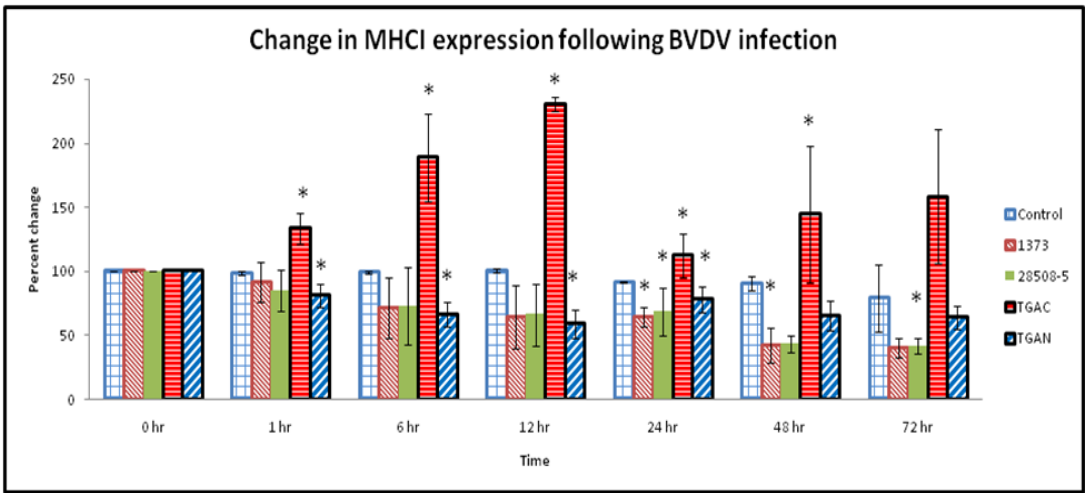

Figure 3-1. Effect of BVDV on MHCI expression on MDDC. Differentiated MDDC were infected with either of four strains of BVDV including high virulent ncpBVDV2a-1373, typical virulent ncpBVDV2a-28508-5 or a homologous pair of ncp or cp type 1b viruses (ncpBVDV1b-TGAN or cpBVDV1b-TGAC) with 6 MOI of infection. The cells were stained for MHCI at1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. The mean fluorescent intensity (MFI) of cells expressing MHC I was analyzed using flow cytometry. Bar showing (*) are significantly different than its time point control ($p<0.05$).

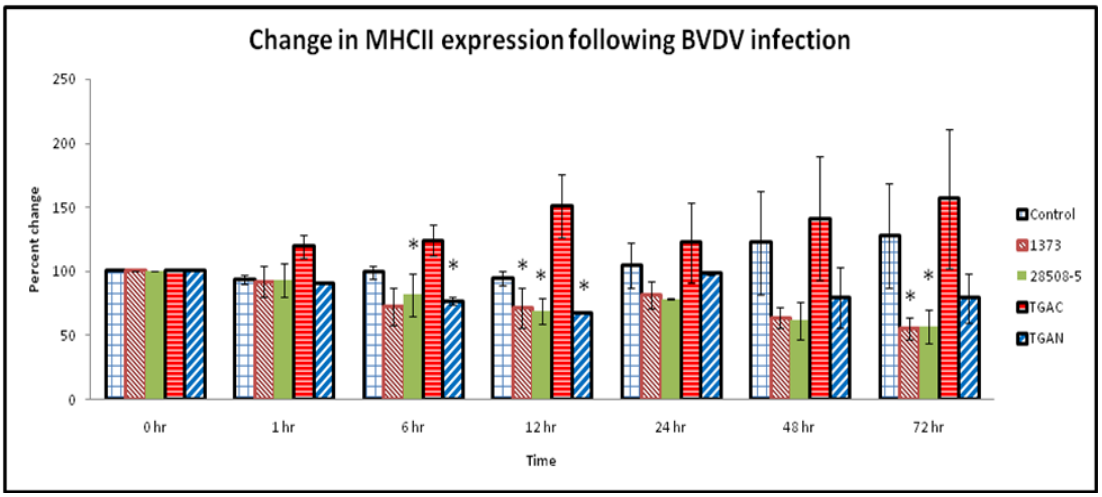

Figure 3-2. Effect of BVDV on MHCII expression on MDDC. Differentiated MDDC were infected with either of four strains of BVDV including high virulent ncpBVDV2a-1373, typical virulent ncpBVDV2a-28508-5 or a homologous pair of ncp or cp type 1b viruses (ncpBVDV1b-TGAN or cpBVDV1b-TGAC) with 6 MOI of infection. The cells were stained for MHCII at1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. The mean fluorescent intensity (MFI) of cells expressing MHC II was analyzed using flow cytometry. Bar showing (*) are significantly different than its time point control ($p<0.05$).

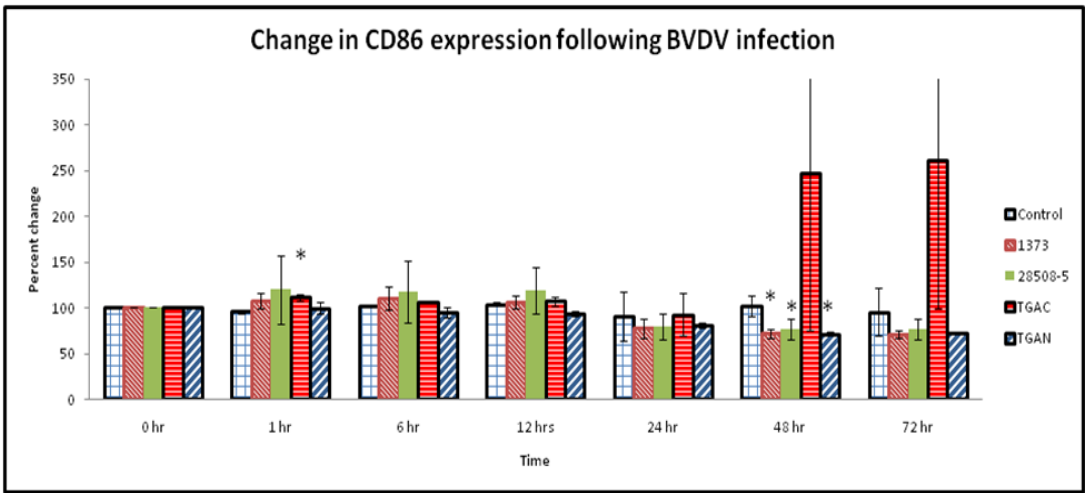

Figure 3-3. Effect of BVDV on CD86 expression on MDDC. Differentiated MDDC were infected with either of four strains of BVDV including high virulent ncpBVDV2a-1373, typical virulent ncpBVDV2a-28508-5 or a homologous pair of ncp or cp type 1b viruses (ncpBVDV1b-TGAN or cpBVDV1b-TGAC) with 6 MOI of infection. The cells were stained for CD86 at1 hr, 6 hr,12 hr, 24 hr, 48 hr or 72 hr p.i. The mean fluorescent intensity (MFI) of cells expressing CD86 was analyzed using flow cytometry. Bar showing (*) are significantly different than its time point control ($p<0.05$).

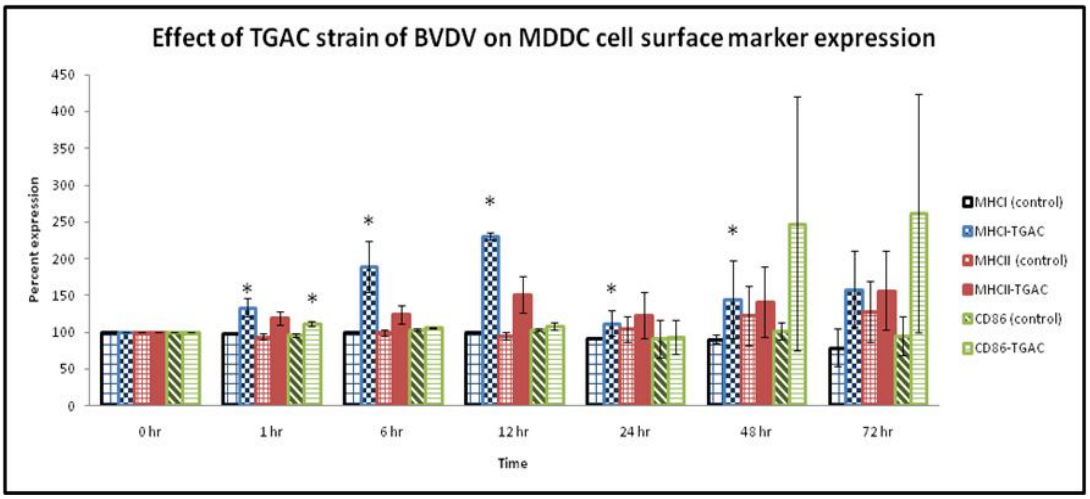

Figure 3-4. Effect of cpBVDV1b-TGAC strain of BVDV on cell surface marker expression of MDDC. Differentiated MDDC were infected with cpBVDV1b-TGAC strain of BVDV with 6 MOI of infection. The cells were stained for MHCI, MHCII or CD86, at1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. The mean fluorescent intensity (MFI) of cells expressing MHCI, MHCII and CD86 was analyzed using flow cytometry. Bar showing (*) are significantly different than its time point control ($p<0.05$).

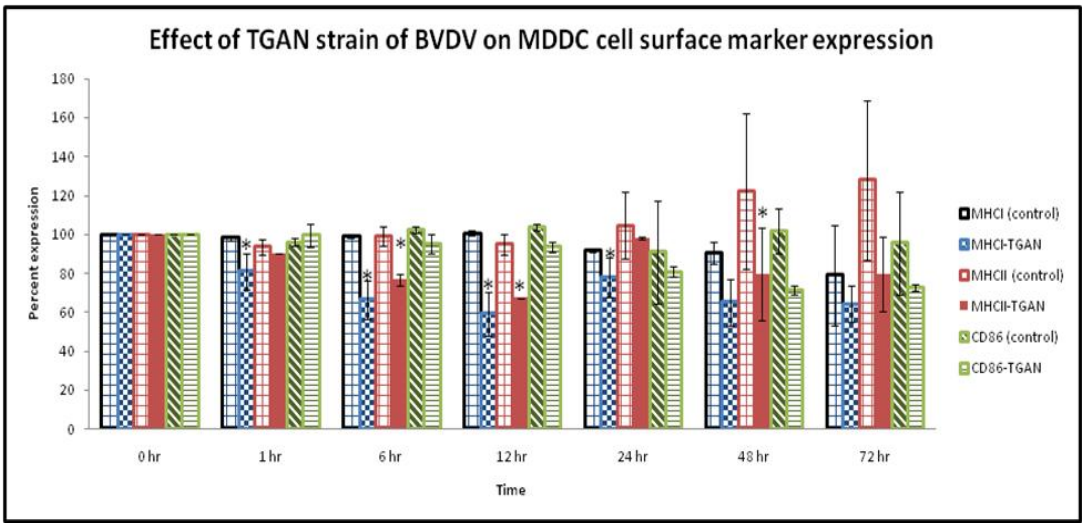

Figure 3-4. Effect of ncp BVDV1b-TGAN strain of BVDV on cell surface marker expression of MDDC. Differentiated MDDC were infected with ncp BVDV1b-TGAN strain of BVDV with 6 MOI of infection. The cells were stained for MHCI, MHCII or CD86 at1 hr, 6 hr,12 hr, 24 hr, 48 hr or 72 hr p.i. The mean fluorescent intensity (MFI) of cells expressing MHCI, MHCII and CD86 was analyzed using flow cytometry. Bar showing (*) are significantly different than its time point control ($p<0.05$).

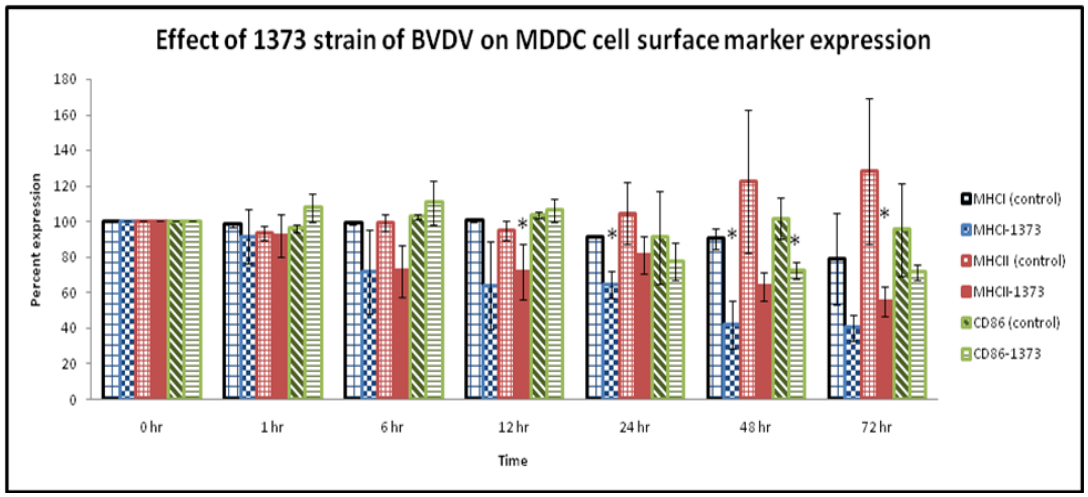

Figure 3-5. Effect of ncp BVDV1b-1373 strain of BVDV on cell surface marker expression of MDDC. Differentiated MDDC were infected with high virulent ncpBVDV2a-1373 strain of BVDV with 6 MOI of infection. The cells were stained for MHCI, MHCII or CD86, at1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. The mean fluorescent intensity (MFI) of cells expressing MHCI, MHCII and CD86 was analyzed using flow cytometry. Bar showing (*) are significantly different than its time point control ($p<0.05$).

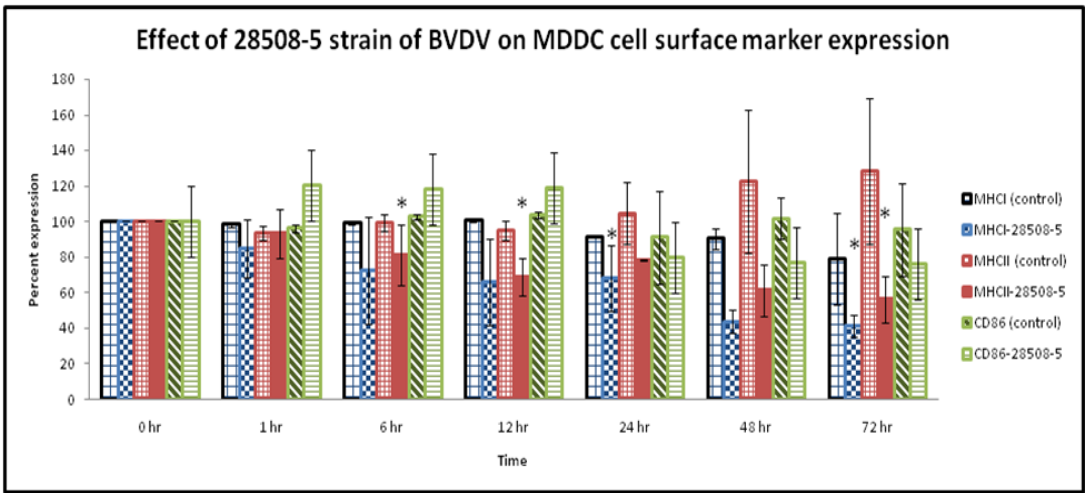

Figure 3-6. Effect of cp BVDV1b-28508-5 strain of BVDV on cell surface marker expression on MDDC. Differentiated MDDC were infected typical virulent ncpBVDV2a-28508-5 with 6 MOI of infection. The cells were stained for MHCI, MHCII or CD86, at1 hr, 6 hr, 12 hr, 24 hr, 48 hr or 72 hr p.i. The mean fluorescent intensity (MFI) of cells expressing MHCI, MHCII and CD86 was analyzed using flow cytometry. Bar showing (*) are significantly different than its time point control ($p<0.05$).

MHCI					
Time	**Control**	**ncpBVDV2a-1373**	**ncpBVDV2a-28508-5**	**cpBVDV1b-TGAC**	**ncpBVDV1b-TGAN**
1 hr	98.32±1.38	86.73±18.33	78.62±17.17	133.5±11.88*	82.15±3.72*
6 hr	99.24±1.09	60.26±18.02	64.05±37.01	189.1±34.30*	70.58±0.76*
12 hr	100.63±1.20	52.31±20.05	57.94±27.84	230.29±5.22*	54.61±2.03*
24 hr	91.44±5.44	64.32±7.41*	68.14±18.62*	112.22±17.29*	77.96±9.93*
48 hr	90.47±5.85	41.85±13.64*	43.57±6.62	144.60±53.14	65.23±11.90
72 hr	78.90±25.86	40.10±7.18	41.30±5.95*	158.1±52.43	64.15±9.25

Table 3- 1. Cell surface expression of MHCI on MDDC following BVDV infection.

Virally infected cell expression was compared to the controls with the controls normalized at 100%. The experiment was repeated three times. The value with (*) representing the significant difference than its time point control ($p < 0.05$).

	MHCII				
Time	**Control**	**ncpBVDV2a-1373**	**ncpBVDV2a-28508-5**	**cpBVDV1b-TGAC**	**ncpBVDV1b-TGAN**
1 hr	93.57±3.91	89.64±15.59	89.9±20.73	119.15±9.23	90.02±0.10
6 hr	99.16±4.71	63.81±1.57	67.67±8.06*	123.96±12.17	76.61±3.09*
12 hr	94.78±5.48	62.79±5.10*	64.11±8.85*	150.49±24.64	67.32±0.006*
24 hr	104.55±17.26	81.15±10.71	78.10±0.38	122.19±31.38	98.27±0.72
48 hr	122.21±40.27	63.65±8.03	61.41±14.62	140.97±48.37	79.78±23.69
72 hr	128.00±40.86	55.10±8.39*	56.44±13.01*	156.83±54.48	79.33±19.13

Table 3- 2. Cell surface expression of MHCII on MDDC following BVDV infection. Virally infected cell expression was compared to the controls with the controls normalized at 100%. The experiment was repeated three times. The value with (*) representing the significant difference than its time point control ($p < 0.05$).

CD86					
Time	**Control**	**ncpBVDV2a-1373**	**ncpBVDV2a-28508-5**	**cpBVDV1b-TGAC**	**ncpBVDV1b-TGAN**
1 hr	95.94±2.13	108.42±11.49	99±10.08	111.40±3.55*	99.48±6.13
6 hr	102.46±1.60	111.14±17.02	98.17±8.96	105.82±0.29	95.09±5.06
12 hr	103.73±1.88	106.47±9.23	106.22±20	107.44±5.08	93.55±2.49
24 hr	90.83±26.16	77.75±10.55	76.61±13.77	92.83±23.47	80.73±2.65
48 hr	101.63±11.5	72.16±4.63*	76.53±10.84*	247.49±172.29	71.26±2.21*
72 hr	95.44±26.22	71.36±4.08	76.31±11.02	261.22±162.00	72.64±11.38*

Table 3- 3. Cell surface expression of CD86 on MDDC following BVDV infection. Virally infected cell expression was compared to the controls with the controls normalized at 100%. The experiment was repeated three times. The value with (*) representing the significant difference than its time point control ($p < 0.05$).

CHAPTER 4.

EFFECT OF BVDV INFECTION ON AUTOPHAGOSOME INDUCTION

ABSTRACT

Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle. The virulence factors responsible for BVDV pathogenesis are not well characterized. One cellular pathway that has been shown to be important for viral pathogenesis in other virus system is autophagy. Autophagy is a cellular process to recycle and maintain the cellular homeostasis and an important defense process of cell against various invading pathogens. Autophagy occurs at low basal levels in almost all cells. It is up regulated in cellular stress including starvation, oxidative stress or infection. Several viruses including flavivirus family have developed the strategies to subvert or use autophagy for their own benefit including replication. The current study was carried out to unravel the relationship between BVDV infection and autophagy induction and to determine the effect of cp and ncp strain of BVDV on autophagosome formation. Stable expressing GFP-LC3 (Green fluorescent protein – Microtubule-associated protein 1A/1B-)-MDBK cell or GFP-LC3-Bt cell lines were created. The virus pair, cp BVDV1b-TGAC and ncp BVDV1b-TGAN were used in the study. The stable-expressing GFP-LC3 cells were infected with either cp. BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV at a MOI of 6. BVDV induced autophagy in MDBK cells as well as in Bt cells, infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV. There was no significant difference between cp or ncp strains of BVDV in autophagy induction. The autophagy

inducing drug, rapamycin enhanced viral production while autophagy inhibiting drug, 3MA suppressed viral production. The co-localization study using, BVDV NS5A and E1 proteins with the autophagosome marker, LC3 revealed that BVDV does not replicate in autophagosomes. The likely function for autophagy with BVDV infection is to support BVDV replication by providing energy for replication.

INTRODUCTION

Autophagy is a normal cellular physiological process where cytoplasmic components are sequestered, enzymatically digested and recycled to maintain cellular homeostasis **(Mizushima, 2007).** This "cellular recycling program" not only provides nutrients to maintain vital cellular functions during fasting but also provides a pathway to eliminate superfluous or damaged organelles, misfolded proteins, and invading microorganisms **(Levine and Kroemer, 2008).** Autophagy occurs at low basal levels in almost all cells. It is rapidly up regulated when cells need to generate intracellular nutrients and energy during starvation or high bioenergetic demands. Autophagy is also up regulated during oxidative stress, infection or protein accumulation (**Kiffin et al., 2006; Dreux et al., 2009; Liu et al., 2010).**

Autophagy also is important in innate and adaptive immune response against a variety of pathogens including bacteria and viruses **(Deretic, 2005; Deretic and Levine, 2009).** The autophagy is induced by various toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors, retinoic acid-inducible gene I (RIG-I)-like receptors, and damage associated molecular patterns (DAMP) such as high-mobility

group box 1 (HMGB1) protein ligation. The interaction of single-stranded RNA with TLR7 was found as the most potent effector in autophagy induction **(Deretic, 2011)**. Autophagy is induced by LPS through a Toll/interleukin-1 receptor domain containing adaptor inducing interferon beta (TRIF) dependent or myeloid differentiation factor 88 (MyD88) independent TLR4 signaling pathway **(Xu et al., 2007).** In adaptive immunity, autophagy increases the antigen recognition through MHCII molecule with enhanced T cell activation and proliferation **(Paludan et al., 2005; Schmid et al., 2007).** Autophagy controls the proliferation and survival of T and B cells **(Li et al., 2006; Miller et al., 2008)**. The knock down of Agt 5 gene **(**autophagy-related gene 5) in mice reduced total thymocytes and peripheral T and B lymphocytes. Agt 5 knockdown mice displayed a dramatic increase in cell death in peripheral CD8+ T lymphocytes and CD4+ and CD8+ T cells failed to undergo efficient proliferation after TCR stimulation **(Pua et al., 2007).** The antimicrobial defense mechanism of autophagy is controlled by Th1 and Th2 polarization. The Th2 cytokines, IL-4 and IL-13 reduced autophagy in Akt or PKB (Protein Kinase) signaling **(Harris et al., 2007)** while Th1 cytokine, IFN gamma enhanced autophagy and suppressed intracellular survival of mycobacterium **(Gutierrez et al., 2004).** Akt or PKB (Protein Kinase B) is a serine/threonine protein kinase that plays an important role in cell proliferation and apoptosis. Despite the effective role of autophagy in immune response, many viruses have developed strategies to subvert or use autophagy for their efficient replication **(Jackson et al., 2005)**. Autophagy is utilized for replication of poliovirus **(Jackson et al., 2005; Taylor and Kirkegaard, 2007)** and

coxsackieviruses (**Wong et al., 2008; Yoon et al., 2008)**. The flaviviridae use autophagosome in two different ways. Hepatitis C virus (HCV) **(Ait-Goughoulte, et al., 2008; Sir et al., 2008; Dreux and Chisari 2009)**, dengue virus (DENV) **(Panyasrivanit et al., 2009; Heaton et al., 2010; McLean et al., 2011)**, and Japanese encephalitis **(Li et al., 2012)** induce autophagy for their replication while West Nile virus (WNV) induces autophagy for cell survival **(Beatman et al., 2012)**. Bovine viral diarrhea virus (BVDV) is a member of the flavivirus family and the pestivirus virus group. BVDV causes great economic loss in cattle industry through its immunosuppression and persistent infection. A unique characteristic of BVDV is the ability of the virus to have two different phenotypes in cell culture: cytopathic (cp) or noncytopathic (ncp) effects. These phenotypes are the result of well characterized genotypic changes that occur in a fatal form of BVDV, mucosal disease (MD). In MD, BVDV pairs exist where the ncp strains has mutated and become a cp strain. The current study was carried out for better understanding the relationship BVDV infection and autophagy induction along with differential effect of cp and ncp BVDV infection and autophagosomes formation.

MATERIALS AND METHODS

Viruses:

The homologous pair of ncp and cp viruses (ncp BVDV1b-TGAN and ncp BVDV1b-TGAC) recovered from an animal that died of mucosal disease were used in the study. The virus stock was prepared in MDBK cells. A 5 ml of $5x10^5$ MDBK cells/ml were seeded in T25 flasks using minimal essential medium (MEM, Gibco BRL, Grand

Island, NY) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml). The cells were grown up to 60-70% confluency. The cells were infected with viruses with 1 MOI of infection. A 0.75 ml of virus was added to each T25 flask and adsorbed for 1 hr at 37°C in a humidified CO_2 incubator. After 1 hr incubation, non-adsorbed virus was removed and cells were washed with sterile PBS. After the washing, 5 ml MEM medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) was added to each flask. The cells were incubated at 37°C in a humidified CO_2 incubator for 4-5 days or up to 70-80% cytopathic effect for TGAC**.** After 4-5 days of incubation, cells were freezed (-80˚C for 15 minutes) and thawed at 25˚C for two cycles to release the virus. The cell debris was pelleted by centrifugation at 3000 rpm for 10 min at 4°C in 15 ml conical tubes. Supernatants containing virus were titrated, aliquoted and stored at -80 °C for further use. The virus titration was done as per method described earlier (**Reed and Munech, 1938**) by by inoculating the sample serially 1 in 10 dilutions in MEM containing MDBK cells. Briefly MDBK cells were detached from tissue culture flask. The number of cells was adjusted to $5x10^5$ cells/ ml. A one hundred-eighty (180) µl cell suspension was added to each well of 96 well plate. A twenty (20) µl virus was added to first row of the plate. The virus was mixed properly with MDBK cells and 20 µl of this dilution was added to next row to achieve 10 fold dilutions. Last two rows were treated as negative control with no virus. The plate was incubated at 37 °C at humidified incubator for next 4 days. The plate was examined every day for cytopathic effect (CPE) of the virus. The highest dilution showing CPE was used

as end point to calculate the proportionate distance (PD). The PD was used to determine the viral concentration (TCID50) as per formula as described earlier (**Reed and Munech, 1938**).

9. Proportionate distance (PD) = (% CPE at dilution above 50%) – (50%)/ (% CPE at dilution above 50%)- (% CPE at dilution below 50%) (e.g. 60-50/60-0= 0.166)

10. Calculation of end point just next to 50% CPE and conversion into – Log (e.g.10^{-6} dilution would be -6)

11. Calculation of $TCID_{50.}$

12. $TCID_{50}$ for 20 µl= $^{(PD+ \text{ - Log dilution above 50\%})}$ (e.g. $1x10^{6.166}$)

The end point for ncp TGAN BVDV was determined by staining the MDBK cells with anti-BVDV Erns antibody (15C5; IDEXX Laboratories, Westbrook, ME, USA) followed by biotinylated rabbit anti mouse IgG (Zymed, Invitrogen Corporation, Frederick, MD, USA) Steptavidin-HRP (Invitrogen Corporation, Camarillo, CA, USA) and AEC reagent (3 amino-9 ethyl-carbazole) (Sigma-Aldrich, St. Louis, MO, USA).The BVDV positive cells were stained red.

Cells:

The BVDV free MDBK cells (passage 95-110) were used in the study. The cells were grown in minimal essential medium (MEM, Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% BVDV free FBS (PPA, Pasching, Austria), penicillin (100

U /ml) and streptomycin (100 µg /ml). MDBK cells were used for viral propagation, virus titration, and transduction with pseudo-lentivirus containing GFP-LC3 gene, and co-localization using indirect immunofluorescence assay.

The BVDV free Bt cells (passage 31-36) obtained from calf from a BVDV antibody- negative dam were used in the study **(McClurkin et al., 1974).** The cells were cultured using the same conditions as described above for MDBK cells. The Bt cells were used for virus growth curve, transduction by pseudo-lentivirus containing GFP-LC3, co-localization and indirect immunofluorescence assay. The 293T cells (passage 42-49) were cultured using the same conditions as described above for MDBK and Bt cells. The 293T cells were used for plasmid transfection and creating of pseudo-lentivirus.

LC3- GFP stabilized cell line:

Autophagy was visualized in green fluorescent protein-(GFP)- Microtubule-associated protein 1A/1B-light chain 3 (LC3) transducted cells. GFP-LC3 plasmid construct was transducted into Bt and MDBK cells using pseudo-lentivirus. The GFP-LC3 gene from addgene plasmid 11546 (kindly provided by Dr. Karla Kirkegaard) **(Jackson et al., 2005)**, was cut between Ndel and BamH1 or ApaLI and BamH1 restriction sites and inserted into lentivirus vector addgene plasmid 17448 (kindly provided by Dr Eric Campeau) **(Campeau et al., 2009)**. To make pseudo-lentivirus, 293T cells were cultured in 6 well plates to 60-70% confluency. The 293T cells were co-transfected with lentivirus vector plasmid containing GFP-LC3 gene with packaging addgene plasmid 22036 (kindly provided by Dr. Didier Trono, GHI, Switzerland) and the

plasmid containing vesicular stomatitis virus G glycoprotein, addgene plasmid 14888 (kindly provided by Dr. Tannishtha Reya) **(Reya et al., 2003).** The lentivirus vector plasmid containing GFP-LC3, packaging plasmid and the plasmid containing VSV-G gene was mixed at a 2:1:1 ratio. One (1) mg DNA was transfected into 293 T cells using 3 µl transfecting reagents (Roche Applied Science, IN, USA). After 72 hr p.i., supernatant and 293 T cells were collected. The collected 293T cells with its supernatant were freezed (-80˚C) and thawed two times as described earlier to release virus. The GFP-LC3 or LentiBrite recombinant virus (EMD Millipore Corporation, Billerica, MA, USA) was transducted into the MDBK cells or Bt cells. The Transducted cells produced green fluoresce after excitation at 395-475 nm wavelength.

***Drugs and virus production*:**

The rapamycin (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 50 nM in MEM (Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% free fetal calf serum (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 µg /ml) was used as the autophagy inducer while 3-methyladenine (3-MA) (Sigma-Aldrich,St. Louis, MO, USA) at a concentration of 10 mM in MEM (Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% free fetal calf serum (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 µg /ml) was used as autophagy inhibitor **(Lee et al., 2008)**. The Bt cell or MDBK cells were grown in 6 well plates. The cells were infected with either cp TGAC or ncp TGAN strain of BVDV at a MOI of 6 . Mock-infected Bt cells or MDBK cells were used as controls. After 1 hr of incubation,

non-adsorbed virus was removed and cells were washed with sterile PBS. After washing, 3 ml medium supplemented with or without rapamycin (50 nM) or 3-MA (10 mM) was added to each well. The cell supernatant was collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr and 72 hr p.i. for virus production. The virus tier at each time point was measured as per method described earlier (**Reed and Munech, 1938**) by by inoculating the sample serially 1 in 10 dilutions in MEM containing MDBK cells

Autophagy measurement:

The GFP-LC3 transducted Bt cell or MDBK cells were cultured in 4 well chamber slides (Thermo scientific, Wilmington, DE, USA) under the same conditions as described above. The cells were infected with either cp TGAC or ncp TGAN strain of BVDV at a MOI of 6. The mock infected cells were used as control. After 1 hr of incubation non-adsorbed virus was removed and cells were washed with sterile PBS. The cells were supplemented with medium containing rapamycin (50 nM) or 3-MA (10 mM MEM) or none. The cells were fixed with 4% paraformaldehyde (PFA) at 12 hr, 24 hr and 48 hr p.i. and mounted with vectashield mounted medium (Vector laboratories, Brlingame, CA, USA).The presence of green fluorescent LC3 dot formation at each time point was counted manually using florescent microscope **(**Olympus, PA, USA) and the percentage of autophagosome positive cells were calculated with following formula Percentage of autophagy = (Number of transducted cell showing autophagy/ Total Number of transducted cell) x100

***Co-localization of Autophagosome and BVDV Proteins*:**

The GFP-LC3 transducted Bt cells were cultured in 4-well chamber slides as described above. The Bt cells were infected with ncpBVDV1b-TGAN strain of BVDV at a MOI of 6. Mock-infected cells were used as controls. The viruses were adsorbed for 1 hr by incubation at 37 °C in humidified CO_2 incubator. After adsorption, cells were washed with sterile PBS. One (1) ml MEM (Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% fetal calf serum (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 µg /ml) was added to each well. The chamber slides were incubated 37 °C in humidified CO_2 incubator for next 48 hr. The BVDV TGAN infected-GFP-LC3 transducted Bt cells were fixed with 4% PFA for 15 minutes at room temperature. The fixed cells were permeabilized using 0.3% Triton in PBS containing 3% BSA by incubating at 37°C for 30 minutes. The permeabilized GFP-LC3 transducted Bt cells were stained with rabbit polyclonal anti-NS5A-BVDV or rabbit polyclonal anti-E1 BVDV antibody (kindly provided by Dr. Julia F Ridpath**,** Ruminant Diseases and Immunology Research Unit, National Animal Disease Center, USDA, Ames, IA, USA) at a concentration of 1:1000 in PBS. The cells were incubated at 37 °C for 1 hr in humidified CO_2 incubator. The cells were washed 3 times with PBS for 2-3 minutes each. The cells were then incubated with anti-rabbit antibody conjugated with rhodamine (Sigma-Aldrich, St. Louis, MO, USA) with concentration of 1:1000 in PBS at 37 °C for 1 hr in humidified CO_2 incubator. After secondary antibody incubation, cells were washed 3 times with PBS and one additional wash with milli Q water. The cells were mounted

with Vectashield mounted medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and examined for BVDV protein and LC3 co-localization using a fluorescent microscope **(**Olympus, PA, USA).

Statistical Analysis:

The experiments to measure autophagsome formation in BVDV infected-GFP-LC3-transducted cells or to measure the effect of autophagosomes on virus production were carried out at least three times. The mean and standard deviation were calculated. The significance difference between each time points was determined by use of the student T test at 0.5 % level of significance

RESULT

Induction of autophagy by cp or ncp strains of BVDV:

The purpose of this experiment was to investigate whether BVDV induced autophagy in BVDV infected cell and determine the ability of cp or ncp strain of BVDV in autophagy induction. The immortal cell line, MDBK cells and primary cell line, Bt cells, were used in this study. The GFP-LC3 transducted MDBK cell or Bt cells were adjust to 5x105 cell/ml in MEM (Gibco BRL, Grand Island, NY) (pH 7-7.4) supplemented with 10% free fetal calf serum (PPA, Pasching, Austria), penicillin (100 U /ml) and streptomycin (100 μg /ml). One ml of this cell suspension was added to each well of4-well chamber slides. The cells were attached to the slide by incubating the cells at 37° C in humidified Co_2 incubator overnight. The cells were infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV with 6 MOI infections. Mock-

infected cells were used as a negative control. After 1 hr virus adsorption, non adsorbed virus was removed and cells were either supplemented with MEM medium containing 10% FBS and antibiotics or MEM medium containing 10% FBS, antibiotics and rapamycin (50 nM) or 3-MA (10 mM).The percentage of transducted cells showing autophagosome as indicated by green fluorescent dots was calculated. The infection of ncp BVDV1b-TGAN or cp BVDV1b-TGAC strain of BVDV induced the autophagy in MDBK cells which was approximately 24%(28.00±3.46%), 50% (72.66±5.03%), 60% (92.66±1.54%) or 22% (26.66±2.3%), 50%(72.00±4.00%), 56% (86.66±1.15%) higher than mock infected time point controls at 12 hr, 24 hr or 48 hr p.i. which were 4.66±1.54%, 23.33±1.54% or 30.66±1.54% at 12 hr, 24 hr or 48 hr p.i. (Table 4-1, Figure 4-1, Figure 4-2, Figure 4-3). The autophagy induction either by ncp BVDV1b-TGAN or cp BVDV1b-TGAC strain of BVDV was significantly different at 24 hr or 48 hr p.i. as compare to time point control ($p< 0.05$). There was no significant difference in induction of autophagy between ncp BVDV1b-TGAN and cp BVDV1b-TGAC strain of BVDV in MDBK cells ($p< 0.05$).The 3MA reduced the autophagy in MDBK cells infected with ncp BVDV1b-TGAN or cp BVDV1b-TGAC strain of BVDV as approximately 15% (13.00±1.00%), 14% (58.00± 4.00%), 28% (64.66±6.11%) or 6% (10.00±5.29%), 14% (66.66±1.15%) and 25% (64.66±2.30%) as compare to MDBK cell infected with ncp BVDV1b-TGAN or cp BVDV1b-TGAC alone at 12 hr, 24 hr and 72 hr p.i. The rapamycin did not increased the production of autophagosomes in MDBK cell infected with cp BVDV1b-TGAC while it increased the autophagosome formation in MDBK cells

infected with ncp BVDV1b-TGAN at as approximately 1% (29.33±2.30%) and 6% (78.66± 12.22%) at 12 hr and 24 hr p.i. than the MDBK cell infected with ncp BVDV1b-TGAN alone (Table 4-1 and Figure 4-2, Figure 4-3).

The effect of BVDV on autophagy induction in Bt primary cell line was measured. The GFP-LC3 transducted Bt cell were infected with ncp BVDV1b-TGAN or cp BVDV1b-TGAC strain of BVDV. The percentage of transducted cells showing autophagosome in form of green fluorescent dots was counted. Both ncp BVDV1b-TGAN or cp BVDV1b-TGAC strains of BVDV induced autophagy that was approximately 7% (17.00±6.92%), 6% (22.66±3.21%), 12% (31.33±7.37%) or 2% (12.66±5.03), 5% (23.33±2.51%) and 9% (28.66±6.43%) higher than mock infected time point controls at 12 hr, 24 hr or 48 hr p.i. which were 10.33±0.58%, 16.66±1.15% and, 19.33±2.88% at 12 hr, 24 hr and 48 hr p.i. respectively (Table 4-2). The supplementation of rapamycin increase the production of autophagosomes in ncp BVDV1b-TGAN or cp BVDV1b-TGAC-infected Bt cells with 7% (24.33±2.08%), 6% (32.00±3.06%), 12% (35.00±3.46%) and 11% (23.00±2.64%), 7% (30.66±4.72%),3% (31.33±4.04%)as compare to Bt cell infected with ncp BVDV1b-TGAN or cp BVDV1b-TGAC at 12 hr, 24 hr and 48 hr p.i. respectively. The 3MA reduced the autophagosome production in ncp BVDV1b-TGAN- or cp BVDV1b-TGAC-infected cells with 6% (11.33±4.94%), 8% (14.33±1.52%), 10% (21.33±2.88%) and 1% (13.00±6.244%), 6% (17.00±4.58%) and 6% (22.00±1.00%) as compare to Bt cell infected with ncp BVDV1b-TGAN or cp BVDV1b-TGAC at 24 hr and 48 hr p.i. respectively (Table 4-2, Figure 4-4, Figure 4-4).

Effect of autophagy on virus production:

The Bt cell or MDBK cells were grown in 6-well plates and infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV at MOI of 6. The mock infected Bt cell or MDBK cells were used as control. After 1 hour incubation non attached virus was removed. The 3 ml medium with or without rapamycin (50 nM) or 3-MA (10 mM) was added to each well. The cell supernatant was collected at 1 hr, 6 hr, 12 hr, 24 hr, 48 hr and 72 hr p.i. for virus production.

There was no virus released in Bt cell supernatant up to 6 hr p.i, infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV. The BVDV production in ncp BVDV1b-TGAN- or cp BVDV1b-TGAC-infected Bt cell was increased from 12 hr p.i. to 48 hr p.i. The Bt cell infected with ncp BVDV1b-TGAN showed the virus production as 0.85±1.20 log10/ml, 1.70±0.00 log10/ml and 3.20±0.70 log10/ml at 12 hr, 24 hr and 48 hr p.i. respectively (Figure 4-6).The Bt cell infected with cp BVDV1b-TGAC strain of BVDV showed the virus production as 0.85±1.20 log10/ml, 2.70±0.00 log10/ml and 4.20±0.70 log10/ml at 12 hr, 24 hr and 48 hr p.i. respectively (Figure 4.7).

The virus production was increased in cell treated with autophagosome inducer drug, rapamycin while it is suppressed by autophagosome inhibitor drug, 3MA in both ncp BVDV1b-TGAN- and cp BVDV1b-TGAC-infected Bt cells. The rapamycin increased one log virus production in ncp BVDV1b-TGAN-infected cells from 1.70±0.00 log10/ml to 2.70±0.00 log10/ml at 24 hr p.i. Similarly, rapamycin increased the cp

TGAC production in cp BVDV1b-TGAC infected Bt cells. The cp BVDV1b-TGAC virus production was increased one log from 0.85±1.20 log10/ml to 1.7±0.00 log10/ml, 2.69±0.00 log10/ml to 3.69±0.00 log10/ml and 4.19±0.70 log10/ml to 5.19±0.70 log10/ml at 12 hr, 24 hr and 48 hr p.i. respectively (Figure 4-7).

The cp BVDV1b-TGAC or ncp BVDV1b-TGAN- infected and 3MA treated Bt cells didn't show any virus production up to 24 hr p.i. These cells produced ncp BVDV1b-TGAN with 1.70±0.00 log10/ml and cp BVDV1b-TGAC as 0.85±1.20 log10/ml at 48 hr p.i. (Figure 4.6, Figure 4-7).

The MDBK cells, infected with ncp BVDV1b-TGAN produced virus as 3.70±0.00 log10/ml and 5.19±0.70 log10/ml at 24 hr and 48 hr p.i. respectively. The autophagosome inducing drug, rapamycin facilitated the release of virus one time point earlier. Virus production in rapamycin-treated cell start at 12 hr p.i. as 1.70±0.00 log10/ml. The virus production in ncp BVDV1b-TGAN infected and rapamycin-treated cell revealed virus titer as 4.20±0.70 log10/ml and 5.70±0.00 log10/ml at 24 hr and 48 hr p.i. respectively that was approximately one log higher at 24 hr and 0.5 log higher at 48 hr than the MDBK cell infected with ncp BVDV1b-TGAN alone

The cell treated with 3MA exhibited suppressed virus production. There was no virus production in MDBK cells infected with ncp BVDV1b-TGAN and supplemented with 3MA up to 24 hr p.i., those cells showed virus production as 1.69±0.00 log10/ml at 48 hr p.i. (Figure 4-8).

The pattern of cp BVDV1b-TGAC virus production was similar in primary (Bt) and immortal (MDBK) cell lines. The MDBK cells, infected with cp BVDV1b-TGAC start producing virus from 12 hr p.i. The virus titer in those cells was reported as 0.85±1.20 log10/ml, 3.70±0.00 log10/ml and 5.70±0.00 log10/ml at 12 hr, 24 hr and 48 hr p.i. respectively. The virus production was increased with autophagy inducing drug, rapamycin while it is suppressed by autophagy inhibiting drug, 3MA. The MDBK cell infected with cp BVDV1b-TGAC and treated with rapamucine revealed virus production as 2.19±0.70 log10/ml, 3.69±0.00 log10/ml, 1.69±0.00 log10/ml at 12 hr, 24 hr and 48 hr p.i. that was two logs higher at 12 hr p.i. and one log higher at 48 hr p.i. as compare to cells that were infected with cp BVDV1b-TGAC alone without rapamycin (Figure 4-9).The MDBK cell infected with cp BVDV1b-TGAC strain of BVDV and treated with 3MA did produce any virus up to 24 hr p.i. and produced virus with titer of 1.69±0.00 log10/ml at 48 hr p.i. that was 4 log lower than the untreated MDBK cells and 5 log lower than MDBK cells treated with rapamycin (Figure 4-9).

Co-localization of autophagy with BVDV proteins:

The various reports of positive strand RNA virus demonstrated the co-localization of autophagosomes and viral proteins, suggested that these viruses replicates in autophagosomes. To determine BVDV replicate in autophagosomes, the co-localization study of LC3 (autophagosome marker) with NS5A, or E1 protein of BVDV was carried out in MDBK cells. None of the above viral proteins were co-localized with LC3, indicating that BVDV did not replicate in autophagosomes (Figure 4.10, Figure 4-11),

while the increase in virus titer with up regulation of autophagosomes indicate that autophagy indirectly facilitated BVDV replication.

DISCUSSION

In the current study, the relationship between BVDV infection and autophagy induction was investigated. The study revealed that both ncp and cp strains of BVDV induced autophagy. The autophagy induction in BVDV infected cells was significantly different than mock-infected cells. There was no significant difference between cp and ncp strain of BVDV in formation of autophagosomes. The autophagy inducing drug, rapamycin, increased autophagy and BVDV replication while autophagy inhibiting drug, 3MA, suppressed autophagy and BVDV replication. Cells infected with flaviviruses often result in persistent infection and cell survival (**Brocket al., 1998; Tonry et al., 2005; Appleret al.; 2010)**. The persistent infection with cell survival supported that flaviviruses may induce autophagy **(Codogno and Meijer, 2005).** There is evidence that IFN regulation may be part of the mechanism of autophagy enhancement of the viral replication. One study with vesicular stomatitis virus (VSV) revealed that the conjugated autophagy gene Atg5–Atg12 negatively regulated type I IFN production by direct association with the retinoic acid-inducible gene I (RIG-I) and IFN-beta promoter stimulator 1 (IPS-1) through the caspase recruitment domains (CARDs) **(Jounai et al., 2007).** It is possible then that reduced type I IFN production may result in enhanced viral replication with BVDV persistent infection. Noncytopathic (ncp) BVDV did not produce sufficient type 1 IFN and cause persistent infection **(Charleston et al., 2001)** with

reduced MHCI expression on antigen presenting cells **(Chase et al., 2004).** In contrast, the absence of autophagy gene Atg 5, resulted in ROS-dependent amplification of RLR (RIG-I-like receptors) signaling and increased type I IFNs **(Tal et al., 2009)**.

Several hypotheses have been proposed that suggest that regulating assembly/metabolism is a mechanism for autophagy to increase virus growth. One hypothesis proposes that the autophagosomes may act as a site of replication for several viruses families including two other positive sense RNA viruses families, Nidovirales and Picornaviridae and also the Flaviviridae. The double membrane autophagosomes provide a scaffold for anchoring and concentrating the replication complexes to prevent the immune response triggered by dsRNA intermediates and "recruit" or hijack certain lipids required for genome synthesis **(Wileman, 2006; Miller and Krijnse-Locker, 2008)**. Recent evidence has suggested that autophagy may contribute to the early phase of viral life cycle **(Reggiori et al., 2010)** and increased viral titer. Autophagy regulates the cellular metabolism. The virus infection leads to an autophagy dependent processing of lipid droplets and triglycerides to release free fatty acids. This results in an increase intracellular beta-oxidation and ATP generation. The energy released by beta-oxidation may be utilized for efficient viral replication **(Heaton and Randall, 2010)**. In the current study, using BVDV NS5A and BVDV E1 proteins with autophagosomes marker LC3 revealed that BVDV did not replicate in autophagosomes while it facilitated the replication of BVDV in infected cells. This indicates that the autophagy machinery likely supported viral RNA replication by providing required energy as reported by **(Dreux et**

al., 2009) in HCV replication. The BVDV cytopathic viruses isolated from cattle with lethal mucosal disease had been found to contain the LC3 gene in the viral genome that allowed specific cleavage of its polyprotein into individual viral proteins presumably by the Atg4 protease **(Meyers et al., 1998)** while the BVDV infected cells, carrying the LC3 gene did not affect the expression of LC3 in cells **(Fricke et al., 2004).** The proper cleavage of BVDV viral polyprotein may contribute to efficient progeny virion generation. Both Dengue-2 and Modoc (a murine flavivirus) infection induced the PI3K-dependent autophagy in MDCK cells. The PI3K-dependent autophagy was mediated by flavivirus NS4A gene. The up-regulation of autophagy markedly enhanced the virus replication and prevented cell death **(McLean et al., 2011)**. Similarly, the expression of NS4B gene of HCV alone is able to induce autophagy via interactions with both the early endosome-associated GTPase Rab5 and a class III phosphoinositide 3-kinase, Vps34 **(Su et al., 2011)**. It will be interesting to know which BVDV viral protein/s is/ are responsible to induce autophagy in BVDV infected cells.

Briefly, this study indicated that both cp and ncp biotypes induced the autophagy in immortal as well as primary cell lines. The autophagy induction by BVDV strains was significantly different than mock-infected cells at 24 hr and 48 hr p.i. There was no difference between cp and ncp biotypes of BVDV in induction of autophagy. The autophagy inducing drug, rapamycin enhanced viral production while autophagy inhibiting drug, 3MA suppressed the viral production suggesting that autophagy facilitate the BVDV replication. The co-localization study using, BVDV NS5A and E1 proteins

with the autophagosome marker, LC3 revealed that BVDV does not replicate in autophagosomes. The likely function for autophagy with BVDV infection is to support BVDV replication by providing energy for replication.

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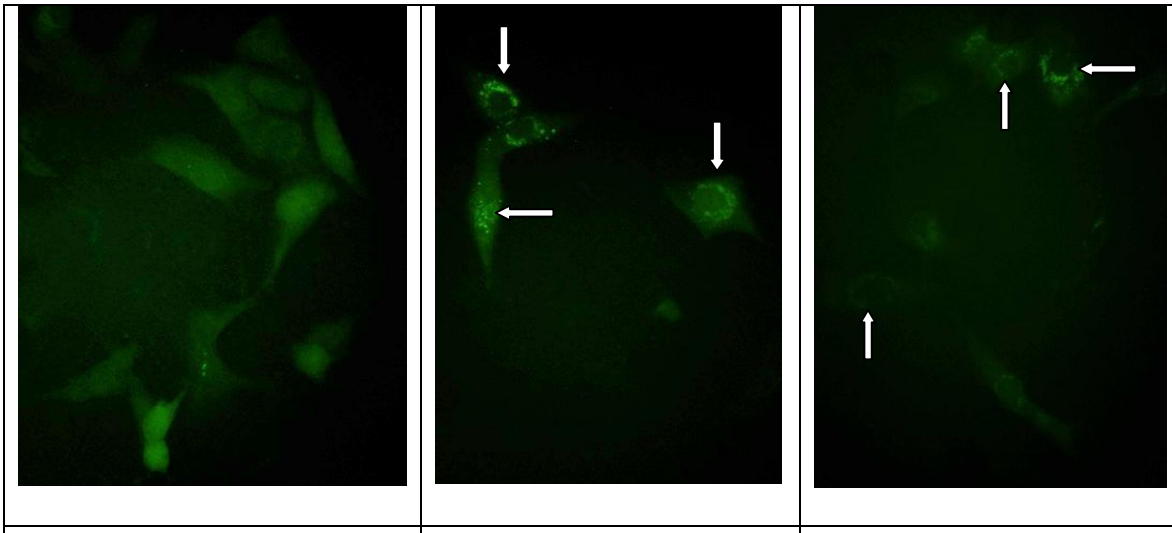

A	B	C

Figure 4-1. Induction of autophagy in MDBK cells after cp BVDV1b TGAC or ncp BVDV1b-TGAN infection. The GFP-LC3 transducted MDBK cells were infected with 6 MOI of either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strains of BVDV for 24 hr. A) Mock-infected control cells; B) cp TGAC or C) ncp TGAN infected MDBK cells showing autophagosome formation. The formation (LC3) of green dots indicated autophagosomes (arrows)

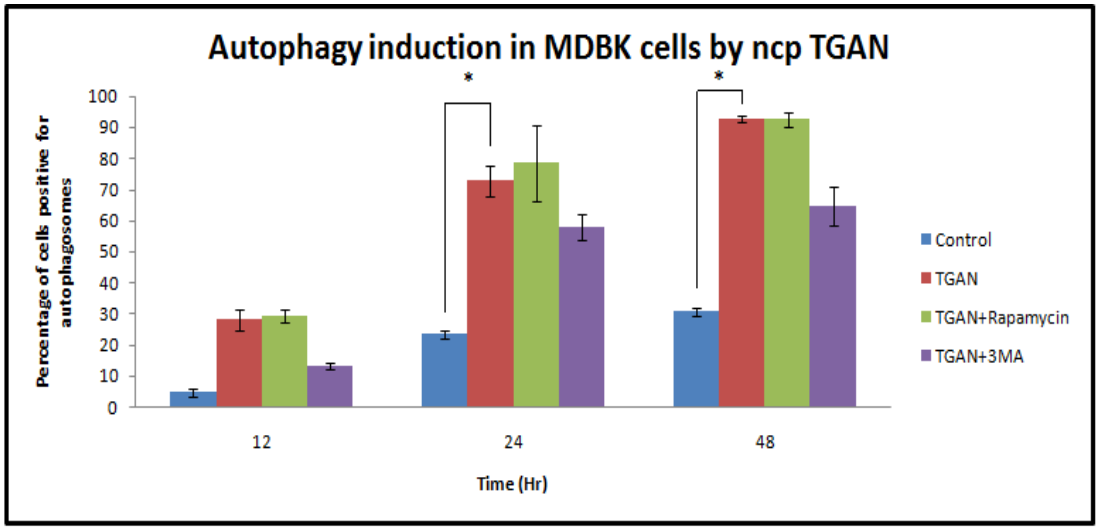

Figure 4-2. The percentage of autophagy in MDBK cells after ncp BVDV1b-TGAN infection. The GFP-LC3 transducted MDBK cells were infected with ncp BVDV1b-TGAN strain of BVDV with MOI of 6. The mock infected cells were used as control. The infected cells were supplemented with either rapamycin (50 nM), 3-MA (10 mM) or none. The percentage of green dots formation (LC3) indicating autophagy in transducted cells were calculated at 12 hr, 24 hr and 48 hr p.i.. The ncpBVDV1b-TGAN-treated cells that induced autophagy significant differences ($p<0.05$) then control at 24 hr or 48 hr p.i. (The significantly different between two treatment is shown by asterix sign {*}, $p<0.05$).

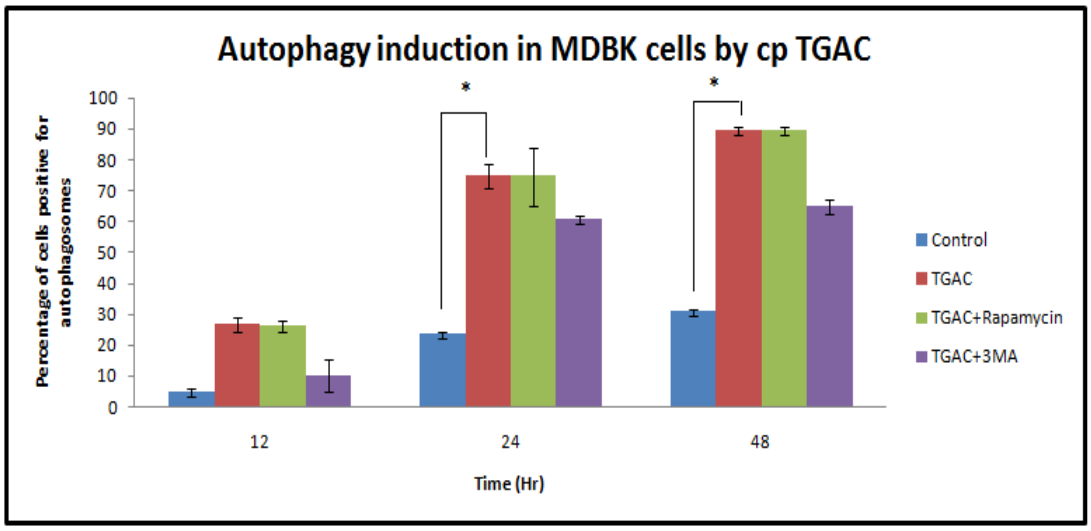

Figure 4-3. The percentage of autophagy in MDBK cells after cp BVDV1b-TGAC infection. The GFP-LC3 transducted MDBK cells were infected with cp BVDV1b-TGAC strain of BVDV with MOI of 6. The mock-infected cells were used as control. The infected cells were supplemented with either rapamycin (50 nM), 3-MA (10mM) or no treatment. The percentage of green dots formation (LC3) indicating autophagy in transducted cells were calculated at 12 hr, 24 hr and 48 hr p.i. respectively. The cpBVDV 1b-TGAC- treated cells induced autophagy significantly then control at 24 hr or 48 hr p.i. (The significantly different between two treatment is shown by asterix sign {*}, $p<0.05$).

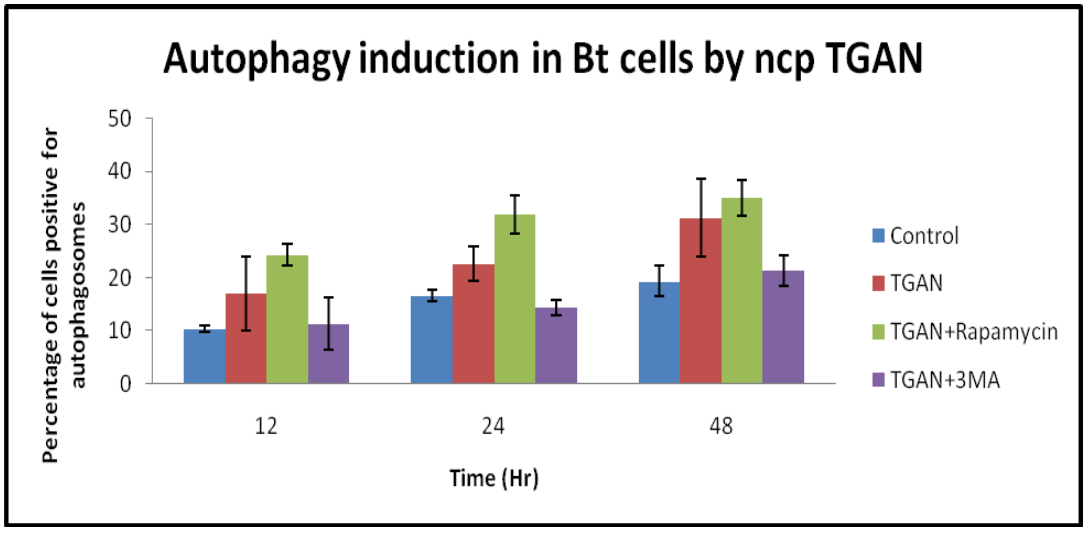

Figure 4-4. The percentage of autophagy in Bt cells after ncp BVDV1b-TGAN infection. The GFP-LC3 transducted Bt cells were infected with ncp BVDV1b-TGAN strain of BVDV with MOI of 6. The mock infected cells were used as controls. The infected cells were supplemented with either rapamycin (50 nM), 3-MA (10mM) or no treatment. The percentage of green dots formation (LC3) indicating autophagy in transducted cells were calculated at 12 hr, 24 hr and 48 hr p.i. respectively.

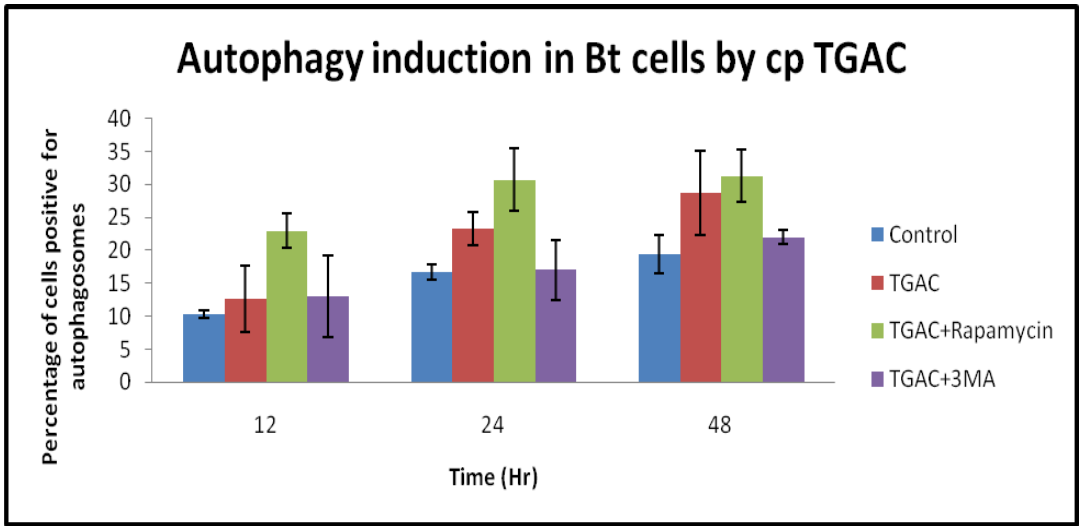

Figure 4-5. The percentage of autophagy in Bt cells after cp BVDV1b-TGAC infection. The GFP-LC3 transducted Bt cells were infected with cp BVDV1b-TGAC strain of BVDV with MOI of 6. The mock infected cells were used as control. The infected cells were supplemented with either rapamycin (50 nM), 3-MA (10 mM) or no treatment. The percentage of green dots formation (LC3) indicating autophagy in transducted cells were calculated at 12 hr, 24 hr and 48 hr p.i. respectively.

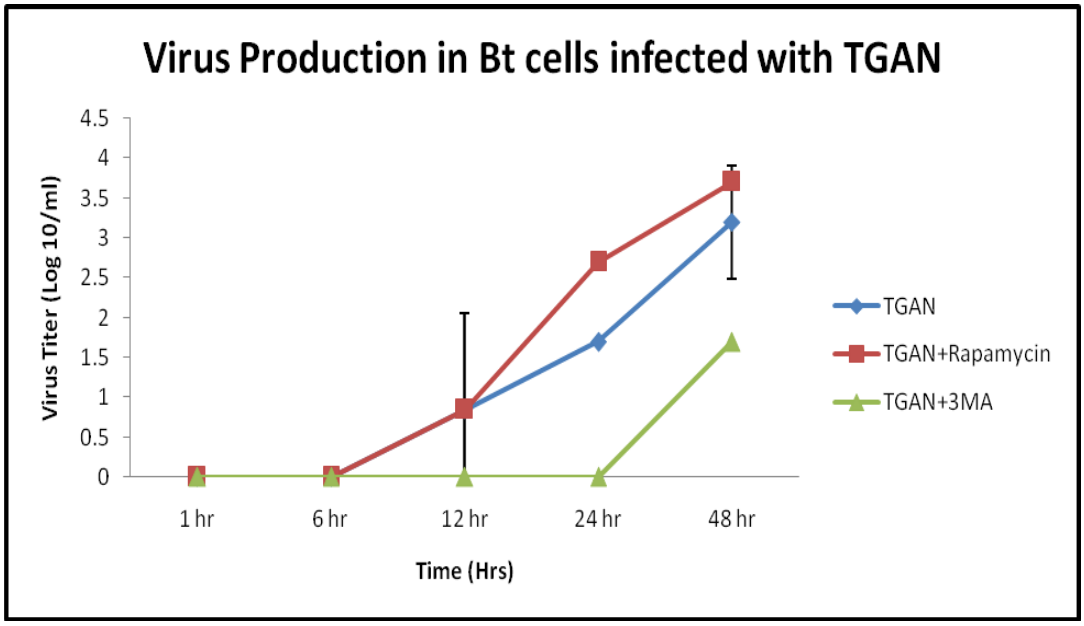

Figure 4-6. The ncp BVDV1b-TGAN virus production by Bt cells in response to autophagy inducing or inhibiting drugs. The Bt cells were infected with cp BVDV1b-TGAN strain of BVDV with MOI of 6. After virus adsorption, the infected cells were washed and supplemented with media containing either rapamycin (50 nM), 3-MA (10mM) or no treatment. The cell supernatant was collected at 1 hr, 6 hr, 12hr, 24 hr or 48 hr p.i. The virus titer was measured at each time point.

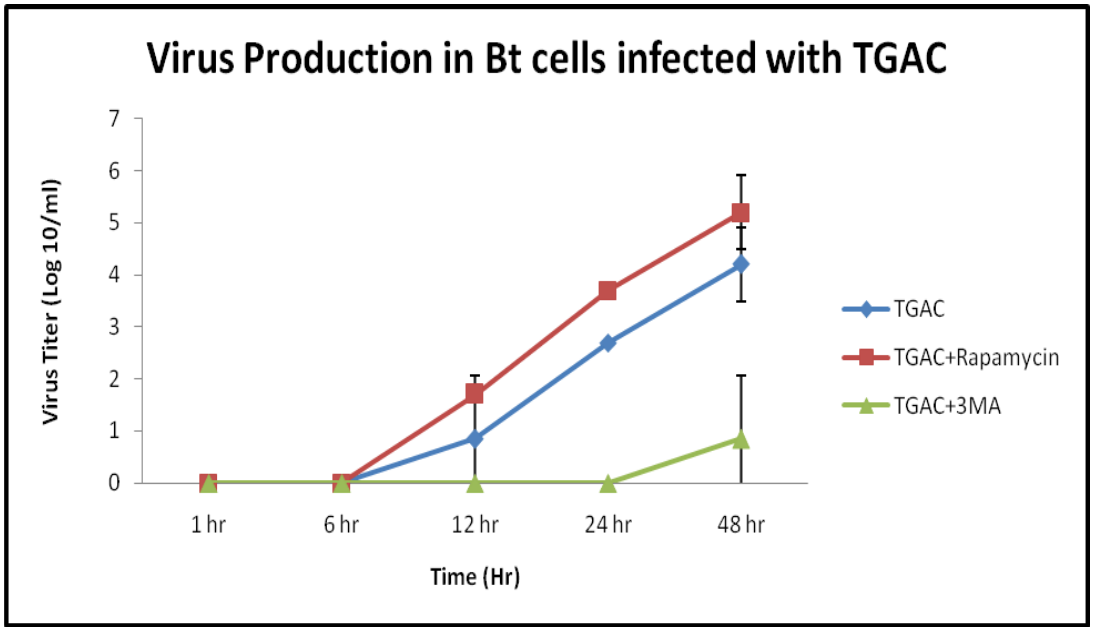

Figure 4-7. The cp BVDV1b-TGAC virus production by Bt cells in response to autophagy inducting or inhibiting drugs. The Bt cells were infected with cp BVDV1b-TGAC strain of BVDV with MOI of 6. After virus adsorption, the infected cells were washed and supplemented with media containing either rapamycin (50 nM), 3-MA (10 mM) or none. The cell supernatant was collected at 1 hr, 6 hr, 12hr, 24 hr and 48 hr p.i. The virus titer was measured at each time point.

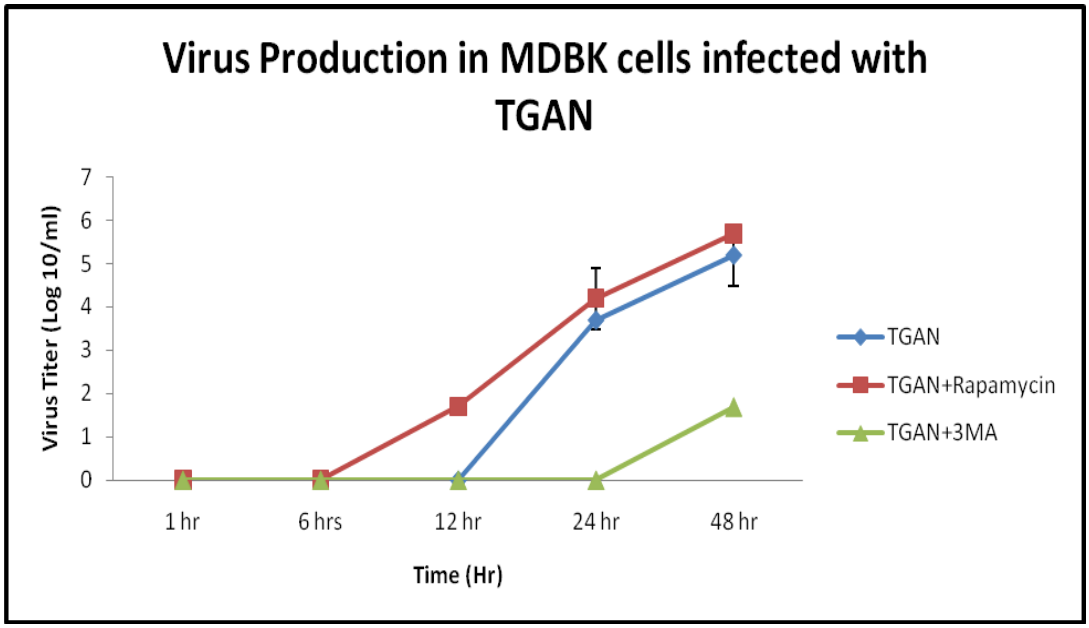

Figure 4-8. The ncp BVDV1b-TGAN virus production by MDBK cells in response to autophagy inducing or inhibiting drugs. The MDBK cells were infected with ncpBVDV1b-TGAN strain of BVDV with MOI of 6. After virus adsorption, the infected cells were washed and supplemented with media containing either rapamycin (50 nM), 3-MA (10mM) or no treatment. The cell supernatant was collected at 1 hr, 6 hr, 12hr, 24 hr and 48 hr p.i. The virus titer was measured at each time point.

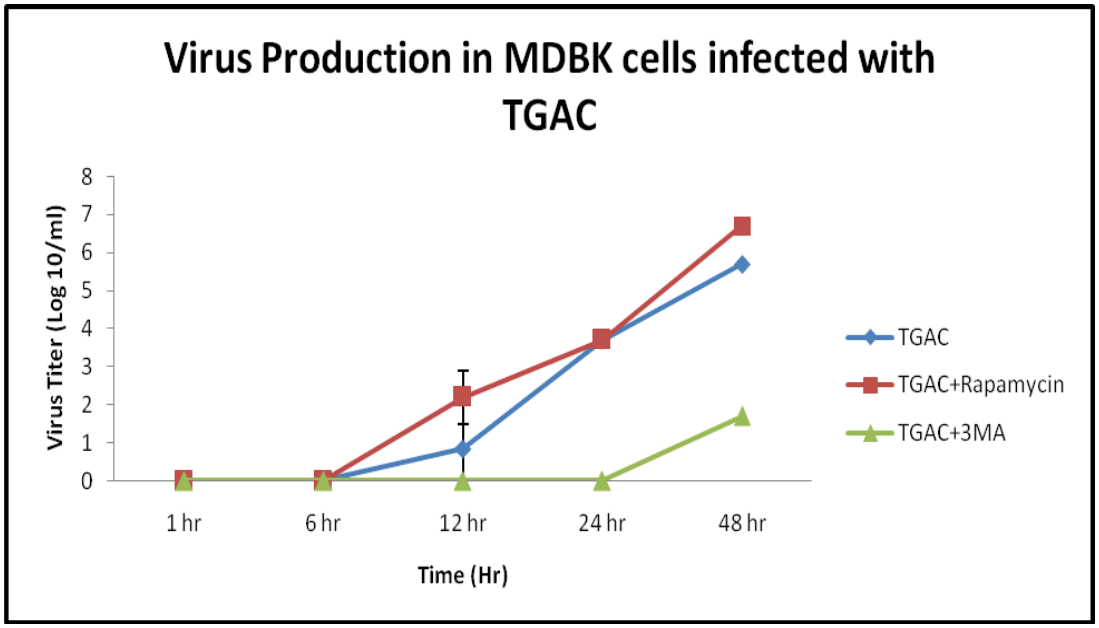

Figure 4-9. The cp BVDV1b-TGAC virus production by MDBK cells in response to autophagy inducing or inhibiting drugs. The MDBK cells were infected with cp BVDV1b-TGAC strain of BVDV with MOI of 6. After virus adsorption, the infected cells were washed and supplemented with media containing either rapamycin (50 nM), 3-MA (10mM) or no treatment. The cell supernatant was collected at 1 hr, 6 hr, 12hr, 24 hr and 48 hr p.i. The virus titer was measured at each time point.

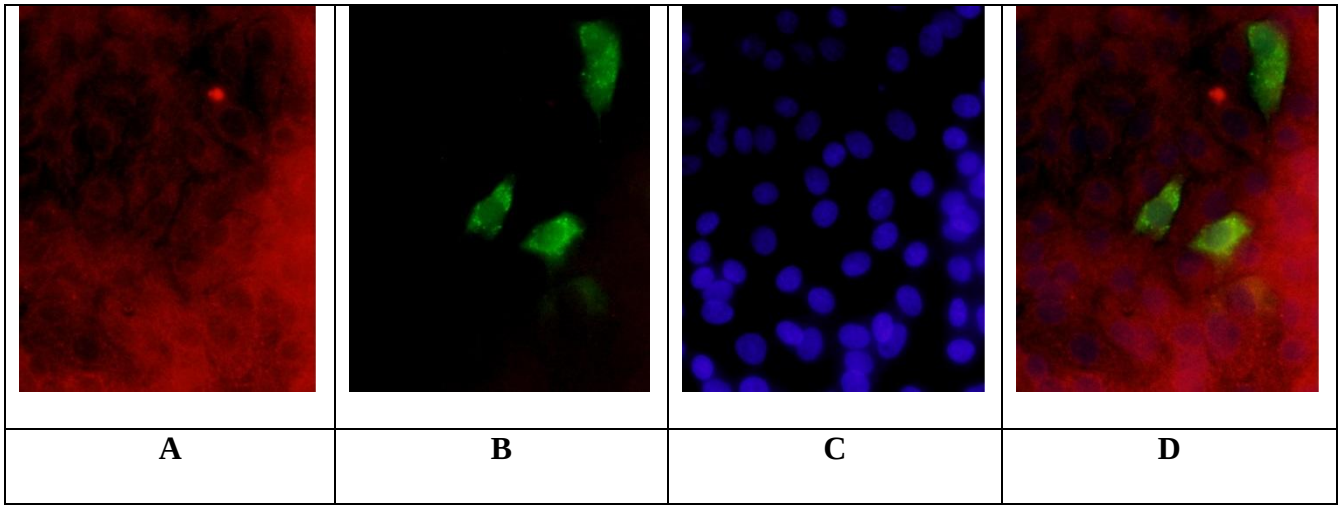

Figure 4-10. The co-localization of BVDV E1 protein and LC3. The GFP-LC3 transducted MDBK cells were infected with ncp BVDV1b-TGAN strain of BVDV with MOI of 6 for 48 hr. After 48 hr infection, cells were fixed and stained with rabbit anti BVDV E1 antibody followed by anti rabbit antibody conjugated with rhodamine. A) BVDV E1 proteins with red; B) LC3 formation in green; C) the blue colored cell nucleus and D) co-localization of BVDV E1 protain and LC3.

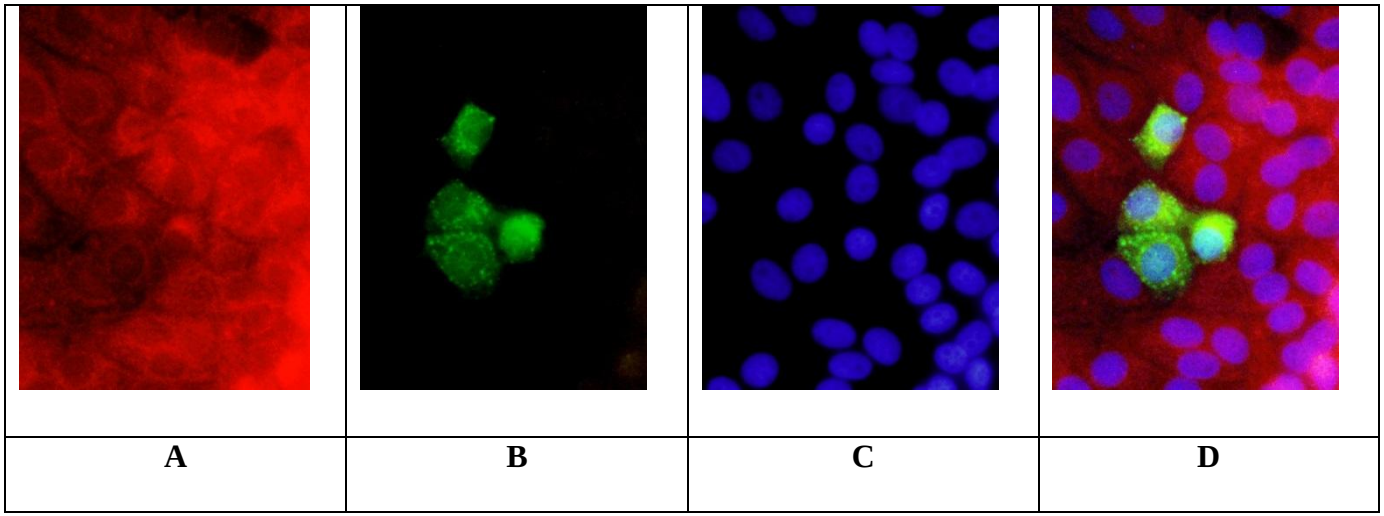

Figure 4-11. The co-localization of BVDV NS5A protein and LC3. The GFP-LC3 transducted MDBK cells were infected with ncp BVDV1b-TGAN strain of BVDV with MOI of 6 for 48 hr. After 48 hr infection, cells were fixed and stained with rabbit anti BVDV NS5A antibody followed by anti rabbit antibody conjugated with rhodamine. A) BVDV NS5A proteins with red; B), LC3 formation in green; C) the blue colored cell nucleus, and D) co-localization of BVDV NS5A protein and LC3.

Time	Control	cpTGAC	cpTGAC+ Rapamycin	cpTGAC+ 3MA	ncpTGAN	ncpTGAN+ Rapamycin	ncpTGAN+ 3MA
12 hr	4.66±1.15	26.66±2.31	26.00±1.63	10.00±5.29	28.00±3.46	29.33±2.31	13.00±1.00
24 hr	23.33±1.15	74.66±4.00	74.66±9.43	60.66±1.15	72.66±5.03	78.66±12.22	58.00±4.00
48 hr	30.66±1.15	89.33±1.15	89.33±1.89	64.66±2.31	92.66±1.15	92.66±2.31	64.66±6.11

Table 4-1. The percentage of autophagy in MDBK cells after cp BVDV1b-TGAC or ncpTGAN infection. The GFP-LC3 transducted MDBK cells were infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV with MOI of 6 with mock-infected control. The infected cells were supplemented with either rapamycin (50 nM), 3-MA (10 mM) or no treatment. The percentage of green dot formation (LC3) indicating autophagy in transducted cells was calculated at 12 hr, 24 hr and 48 hr p.i. respectively.

Time	Control	cpTGAC	cpTGAC+ Rapamycin	cpTGAC+ 3MA	ncpTGAN	ncpTGAN+ Rapamycin	ncpTGAN+ 3MA
12 hr	10.33±0.57	12.66±5.03	23±2.64	13±6.24	17±6.92	24.33±2.08	11.33±4.93
24 hr	16.33±1.15	23.33±2.51	30.66±4.72	17±4.28	22.66±3.21	32±3.60	14.33±1.52
48 hr	19.33±2.88	28.66±6.42	31.33±4.04	22±1.00	31.33±7.37	35±3.46	21.88±2.88

Table 4-2. The percentage of autophagy in Bt cells after cp. BVDVb1-TGAC or ncp BVDVb1-TGAN infection. The GFP-LC3 transducted Bt cells were infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV with MOI of 6 with mock-infected control. The infected cells were supplemented with either rapamycin (50 nM), 3-MA (10 mM) or no treatment. The percentage of green dot formation (LC3) indicating autophagy in transducted cells was calculated at 12 hr, 24 hr and 48 hr p.i. respectively

CHAPTER 5.

EFFECT OF ACUTE BVDV INFECTION ON ANTIBODY LEVELS AND POLARITY OF THE HUMORAL IMMUNE RESPONSE

ABSTRACT

Bovine viral diarrhea virus (BVDV) infection causes transient immunosuppression in acutely infected animals. The study was conducted to investigate the effect of cytopathic (cp) or noncytopathic (ncp) strain of BVDV in immunosuppression and polarization of immune response. The concentrations of total IgG, IgG1 and IgG2 were used to estimate the T helper -2 (TH-2) and T helper -1 (TH-1) immune responses. Fourteen, 5-6 months old BVDV seronegative calves were divided in to two groups with seven calves each. One group was infected with ncp BVDV1b-TGAN strain of BVDV while the other group was infected with the member of the pair, cp BVDV1b-TGAC strain intranasally. Blood was collected at 0, 7, 14, 21 and 35 days post infection (p.i.). The serum was separated and the concentration of total IgG, IgG1 and IgG2 was measured by ELISA. The concentrations of total IgG and IgG1 were reduced by 40-60%by day 7 p.i. in both the groups. The concentrations of total IgG and IgG1gradually increased from day 7 to day 35 in both the groups. The IgG2 concentration fluctuated in both the groups throughout the study.

The IgG2 concentration increased significantly (doubled) from day 0 to day 7 in calves infected with ncp BVDV1b-TGAN strain of BVDV than was reduced by about 30% at 21 days p.i. and then increased at 35 days to the level seen at day 7. The

concentration of IgG2 at day 35 was almost twice the initial IgG2 concentration in calves infected with ncp BVDV1b-TGAN strain of BVDV and the IgG1/IgG2 ratio was 0.94±0.43, (normal 1.1-1.2) indicating that ncp BVDV1b-TGAN appears to direct the immune response toward a stronger T helper-1 (TH-1) or cellular immune response.

The IgG2 concentration in calves infected with cp BVDV1b-GAC strain of BVDV significantly reduced about 10% from 0 day to 14 days p.i. and then increased significantly approximately 40% at 21 days and reduced again at 35 days to day 14 levels that was lower than the initial concentration of IgG2. The IgG1/IgG2 ratio at 35 days p.i. was 1.34±0.54. The reduced concentration of IgG2 at 35 days p.i. in calves infected with cp BVDV1b-TGAC strain of BVDV indicated that cytopathic BVDV divert the immune response toward T helper -2 (TH-2) or humoral immune response.

The reduction in total IgG following BVDV infection in both the groups suggested that BVDV caused transient humoral immunosuppression following infection. The results of this study supported that the infection of calves with bovine virus diarrhea virus (BVDV) is a transient and self-limiting infection that can result in a period of humoral immunosuppression with the type of TH response affected dependent up on the viral strains.

INTRODUCTION

BVDV is one of the important disease of ruminants in USA and worldwide. BVDV induce immunosuppression at all levels of adaptive response from antigen presentation to B cell apoptosis **(Chase, 2013).** BVDV infection affects the number of

circulating T-lymphocytes and B-lymphocytes. The effect of BVDV infection on the number of circulating T-lymphocytes is strain dependent and varies from a mild lymphopenia (10-20%) with ncp type 1b NY-1 **(Brodersen et al., 1999; Ellis et al., 1988)** to 40-50% with ncp type 1b R2360 **(Ridpath et al., 2007)** to a severe lymphopenia (50-70% decrease) with ncp type 2 field isolate 24515 **(Archambault et al., 2000)** or ncp type 1b CA0401186a **(Ridpath et al., 2007).** The number of circulating B-lymphocytes also decreased following infection with ncp NY1 BVDV from days 3 to 12 **(Ellis et al., 1988)** while other studies did not found any effect on circulating B-lymphocytes following infection with ncp type 2 field isolate 24515 **(Archambault et al., 2000).**

BVDV infections have their major effect on follicular B-lymphocytes. In the lymphoid tissue, depletion of B cells occurs in the lymphoid follicles of the lymph nodes with highly virulent ncp BVDV and in Peyer's patches with both mucosal disease and highly virulent BVDV infections **(Chase, 2013).** Circulating B lymphocytes have a direct effect on antibody synthesis and induction of humoral immune response. Intrabronchial BVDV infection with Singer strain increased, BVDV-specific IgA in bronchial alveolar fluid (BAF). The IgA in bronchial alveolar fluid increased to four-fold greater than background titers by day 7 post-primary infection, and to levels 20-fold above background within 7 days of the secondary viral infection **(Silflow et al., 2005).**

The development of active immunity eliminates the virus and virus is rarely detected in buffy coat cells or nasal secretions **(Howard et al., 1989).** The current study

was done to evaluate the effect of cp and ncp BVDV on immunosuppression and polarization of immune response in postnatal calves. The polarization of immune response was measured indirectly through concentrations of IgG1 and IgG2 to estimate the TH2 and TH1immune responses respectively **(Kawasaki et al., 2004)**

MATERIALS AND METHODS

Animals and viral infection:

Fourteen (14) Holstein Friesian calves (5-6 months of age) were divided in to two groups with seven calves each, housed at in separate isolated pens, at SDSU, Brookings, SD, U.S.A.. The SDSU Institutional Animal Care and Use Committee approved the animal protocol procedures. All animals were healthy and seronegative for BVDV. A homologous pair of ncp and cp type 1b viruses Tifton GeorgiA Non-cytopathic (TGAN) or Tifton GeorgiA Cytopathic virus (TGAC) recovered from an animal that died of mucosal disease **(Brownlie et al., 1984; Fritzemeier et al., 1995; Ridpath et al., 1991)** were used to infect the animals. One group was infected with cp BVDV1b-TGAN strain of BVDV while the other group was infected with the other partner of the pair, cp BVDV1b-TGAN strain of BVDV. A total volume of 4 ml ($1x10^6$pfu/ ml) virus was administered intranasally for each animal. Blood was collected at 0, 7, 14, 21 and 35 days p.i. The serum was separated and analyzed for total IgG, IgG1 and IgG2 concentrations via ELISA.

Measurement of Total IgG Serum:

The serum concentration of total IgG was measured by bovine IgG ELISA kit (Bethyl Laboratories Inc, Montgomery, TX, USA), The 50 mMTris buffer containing 0.14% M NaCl and 0.05% tween, pH 8.0 was used as sample and standard diluting buffer, blocking buffer and wash buffer as per the manufacture's guidelines. To perform the ELISA for total IgG, the serum was diluted 1:160,000 in sample diluting buffer. The standard of known concentration for IgG was serially diluted in standard diluting buffer to achieve the concentrations of IgG of 10,000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.625 ng/ml and 7.8 ng/ml. The ELISA plates were coated with 100µl IgG capture antibody diluted in 0.05 M carbonate- bicarbonate buffer (pH 9.6). The plates were incubated with capture antibody at room temperature for 60 minutes. Following coating, the plates were washed three times with wash buffer and blocked with 200 µl blocking buffer for 30 minutes at room temperature. The ELISA plates were washed three times with wash buffer. The 100 µl serially diluted IgG standards and samples were added to different wells of ELISA plates along with blank. The plates were incubated at room temperature for 60 minutes. The plates were washed five times with wash buffer. Following washing, 100 µl of horseradish peroxidase (HPR) (1:22,500 dilution in HRP diluting buffer from the ELISA kit) was added to each well. The plates were incubated for 60 minutes at room temperature and washed five times with wash buffer. One hundred (100) µl substrate was added to each well as per manufacture's guidelines. The plates were incubated in the dark for 15 minutes at room

temperature. The reaction was stopped using 2M H_2S0_4 and the OD value was measured by Biotek ELx808 ELISA plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm wavelength. The standard curved was plotted using Soft-Max pro software. The concentration of total IgG for each sample was estimated using the standard curve.

Measurement of IgG1 Serum:

The concentration of IgG1 in serum was detected by using a bovine IgG1 ELISA kit (Bethyl Laboratories Inc, Montgomery, TX, USA). The 50 mM Tris buffer containing 0.14% M NaCl and 0.05% tween, pH 8.0 was used as HRP, sample and standard diluting buffer as well as blocking and wash buffer as per the manufacture's guidelines. The serum was diluted in sample diluting buffer to achieve final dilution of 1:80,000. The IgG1 standard was serially diluted in standard diluting buffer to achieve the concentrations of 10,000 ng/ml, 1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml and 15.625 ng/ml.

The ELISA plates were coated with 100µl IgG1 capture antibody diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plates were incubated at room temperature for 60 minutes. Following coating, the plates were washed three times with wash buffer and blocked with 200 µl blocking buffer for 30 minutes at room temperature. The ELISA plates were washed three times with wash buffer. The 100 µl serially diluted IgG1 standards and samples were added to different wells of ELISA plate along with blank. The plates were incubated for 60 minutes at room temperature. The plates were washed

five times with wash buffer. Following washing, 100 µl HPR (1:22,500 diluted in HRP diluting buffer) was added to each well. The plates were incubated for 60 minutes at room temperature and washed five times with wash buffer. The 100 µl substrate was added to each well. The plates were incubated for 15 minutes in the dark at room temperature. The reaction was stopped by adding 100 µl 2M H_2S0_4 in each well. The OD value for each well was measured by Biotek ELx808 ELISA plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm wavelengths. The standard curved was plotted using Soft-Max pro software and concentration of IgG1 for each sample was estimated using the standard curve.

Measurement of IgG2 Serum:

The concentration of IgG2 in the calves' serum was measured by using a bovine IgG2 ELISA kit (Bethyl Laboratories Inc, Montgomery, TX, USA). The 50 mM Tris buffer containing 0.14% M NaCl and 0.05% tween, pH 8.0 was used as sample, standard and HPR diluting buffer as well as blocking and wash buffer as per the manufacture's guidelines. To perform the ELISA for total IgG2, the serum was diluted to 1:80,000 in sample diluting buffer. The IgG2 standard was serially diluted in standard diluting buffer to achieve the concentrations of IgG2 of 10,000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.625 ng/ml and 7.8 ng/ml. The ELISA plates were coated with 100µl IgG2 capture antibody diluted in 0.05 M carbonate- bicarbonate buffer (pH 9.6). The plates were incubated with the capture antibody for 60 minutes at room temperature. The plates were washed three times with wash buffer and blocked with 200

µl blocking buffer for 30 minutes at room temperature. Following blocking, the ELISA plates were washed three times with wash buffer. The 100 µl serially diluted IgG2 standards and samples were added to different wells of ELISA plates along with blank. The plates were incubated at room temperature for 60 minutes. The plates were washed five times with wash buffer. Following washing, 100 µl HPR (1:50,000 in HRP diluting buffer) was added to each well. The plates were incubated for 60 minutes at room temperature and washed five times with wash buffer. The 100 µl substrate was added to each well as per manufacture's guideline. The plates were incubated at dark for 15 minutes at room temperature. The reaction was stopped by adding 100 µl, 2M H2S04. The OD value was measured by Biotek ELx808 ELISA plate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 450 nm wavelength. The standard curved was plotted using Soft-Max pro software. The concentration of IgG2 for each sample was estimated using a standard curve.

Statistical analysis:

The mean concentration of IgG, IgG1 and IgG2 was calculated. The variations in results were measured by standard deviation at each time points. The significance difference in IgG, IgG1, IgG2 concentration or IgG1/IgG2 ratio at each time point as well as between the cp and ncp BVDV groups were calculated through student's T test at 5% (0.05) and 10% (0.1) level of significance **(Glantz, 2002).**

RESULTS

Concentration of total IgG:

The concentration of total IgG in serum of calves infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN strain of BVDV was measured at 0, 7, 14, 21 and 35 days p.i. In both groups the concentration of total IgG was significantly ($p<0.05$, $p<0.1$) reduced by 7 days p.i. The initial concentration of total IgG in calves infected with cp BVDV1b-TGAC strain of BVDV was 16.14±1.46 mg/ml that reduced significantly ($p<0.05$, $p<0.1$) 43% to 9.00±0.75 mg/ml at 7 days p.i. The total IgG concentration in calves infected with cp BVDV1b-TGAC strain of BVDV increased from day 7 p.i. to day 35 p.i. At day 14 the IgG concentration was 13.62±0.87 mg/ml, that was 50% more than at 7 days p.i. The concentration of IgG remained almost same at day 21 (14.68±1.38 mg/ml) and then increased significantly ($p<0.05$, $p<0.1$) 26.66±2.56 mg/ml at 35 days p.i. (Table 5-1, Figure 5-1, Figure 5-3). Similarly, the concentration of total IgG in calves infected with ncp BVDV1b-TGAN was significantly reduced ($p<0.05$, $p<0.1$) from 14.17±1.44 mg/ml initial to 10.35±1.11 mg/ml at 7 days p.i. then increased around 30% from at 7 days p.i. to at14 days p.i. (13.17±1.58 mg/ml). The concentration of total IgG remained almost same between 14 days p.i. to 21 days p.i (14.09±1.33 mg/ml) then increased to 19.78±2.43 mg/ml at 35 days p.i. (Table 5-2, Figure 5-1, and Figure 5-3).

Concentration of IgG1:

The concentration of serum IgG1 in calves infected with either cp BVDV1b-TGAC or ncp BVDV1b-TGAN was measured at 0, 7, 14, 21 and 35 days p.i. The cp

BVDV1b-TGAC infection significantly reduced ($p<0.05$, $p<0.1$) the serum IgG1 concentration ~62% (3.95±0.27 mg/ml) at 7 days of infection compared to the initial concentration of 10.75±1.3 mg/ml (Table 5-1). The concentration of IgG1 increased ~54% from day 7. (3.95± 0.27 mg/ml) to day 14 (6.08±0.6 mg/ml) in cp BVDV1b-TGAC infected calves. As was seen with total IgG, the concentration of IgG1 in serum increased slightly from day 14 day (6.08±0.6 mg/ml) to day 21 days (6.68±2.94 mg/ml) and significantly increased ($p<0.1$) from 21 days p.i. to 35 days (9.98±1.18 mg/ml) .p.i. in calves infected with cp BVDV1b-TGAC strain of BVDV. At 35 days p.i. the level of IgG1 was similiar to initial concentration at day 0 (10.75±1.3 mg/ml) (Table 5-1, Figure 5-1, and Figure5-2).The ncp BVDV1b-TGAN infection also reduced serum IgG1 concentration. The serum IgG1 concentration was significantly reduced ($p<0.05$, $p<0.1$) ~60% from 12.93±2.00 mg/ml at initial infection to 7.26±1.18 mg/ml at 7 days p.i (Table 5-2). The serum IgG1 concentration increased 30% from 7.26±1.18 mg/ml at 7 days p.i.to9.71±1.18 mg/ml at 14 days p.i. There was little change in serum IgG1 concentration from 14 days p.i. (9.71±1.18 mg/ml) to 21 days p.i. (9.05±1.66 mg/ml) in calves infected with ncp BVDV1b-TGAN strain. The serum IgG1 concentration returned to levels seen prior to infection at 35 days p.i. (12.91±1.77 mg/ml) in calves infected with ncp TGAN strain of BVDV (Table5-2, Figure 5-1, Figure 5-2).

Concentration of IgG2:

The concentration of IgG2 fluctuated in both the groups, infected with either ncp BVDV1b-TGAN or cpBVDV1b-TGAC strain of BVDV. The calves infected with

cpBVDV1b-TGAC strain of BVDV showed significant reduction ($p<0.05$, $p<0.1$) in IgG2 concentration from 11.44±2.17 mg/ml initial to 10.48±3.94 mg/ml at 7 days p.i. The concentration of IgG2 remained almost the same at 14 days p.i. (10.32±2.02 mg/ml) and then significantly increased ($p<0.05$, $p<0.1$) almost 36% (14.09±2.00 mg/ml) at 21 days p.i. The IgG2 concentration showed its minimum level at 35 days p.i. as 8.50±3.39 mg/ml in calves infected with cpTGAC strain of BVDV (Table 5-1, Figure 5-1, Figure 5-4).

The calves infected with ncp BVDV1b-TGAN strain of BVDV showed different dynamics for IgG2 concentration than the calves infected with cpBVDV1b-TGAC strain of BVDV. The concentration of IgG2 in calves infected with ncp BVDV1b-TGAN strain of BVDV significantly increased ($p<0.05$, $p<0.1$) IgG2 concentration from 7.20±3.27 mg/ml initial to 15.88±3.59 mg/ml at 7 days p.i., which was almost double the initial level. The concentration of IgG2 reduced from 7 days p.i. to 21 days p.i. The concentration of IgG2 reduced approximately 20% from 7 days p.i. (15.88±3.59 mg/ml) to 14 days p.i. (12.75±4.00 mg/ml) and further reduced to 11.54±4.03 mg/ml at 21 days p.i. The concentration of IgG2 increased from day 21 p.i. (11.54±4.03 mg/ml) to 35 days p.i. as 14.00±2.42 mg/ml that was almost double than the initial concentration of IgG2 (7.20±3.27 mg/ml) in calves infected with ncpBVDV1b-TGAN strain of BVDV.

IgG1/IgG2 ratio:

The ratio of serum IgG1 concentration and IgG2 concentration in calves infected with either ncp BVDV1b-TGAN or cp bvdv1B-TGAC strain of BVDV was performed to

determine the diversion of immune response to either T helper-2 (TH-2) or T helper -1 (TH-1) arm.

The IgG1/IgG2 ratio in calves infected with ncp BVDV1b-TGAN strain of BVDV remained low than the initial up to 35 day p.i. The IgG1/IgG2 ratio in calves infected with ncp BVDV1b-TGAN strain of BVDV showed as 1.11±0.11, 0.46±0.24, 0.90±0.65, 0.83±0.65 or 0.94±0.43 at 0, 7, 14, 21 or 35 days p.i.

The IgG1/IgG2 ratio in calves infected with cp BVDV1b-TGAC strain of BVDV reduced at 7 days p.i. than increased up to 35 days p.i. The IgG1/IgG2 ratio in calves infected with cpTGAC strain of BVDV revealed as 0.99±0.44, 0.52±0.47, 0.63±0.16, 0.80±0.59 or 1.34±0.54 at 0, 7, 14, 21 or 35 days p.i.

DISCUSSION

It is well know that acute BVDV infection results in transient leukopenia with decreases in absolute numbers of circulating B lymphocytes, T lymphocytes and neutrophils**(Bolin et al. 1985)**. Reduced circulating B lymphocytes may result in transient reduction in total IgG and IgG1 concentration in BVDV infected animals.

Study related to tissue distribution of BVDV following BVDV infection(cp or ncp or both) in colostrum-deprived or colostrum-fed calves indicated that BVDV localized in lymphoid and gastrointestinal system to induce effective immune response (Spagnuolo-Weaver et al., 1997) while BVDV infected APC have reduced Fc and C3 receptor expression that reduced antigen uptake and presentation **(Welsh et al., 1995)** and fail to induce sufficient immune response.

The ncp BVDV reduced MHC II expression **(Archambaultet al., 2000)**. The reduced antigen uptake and presentation through MHCII may reduce the T helper cell activation that may result in reduced B cell proliferation. Reduced B cell proliferation may be the reason for low IgG production in calves infected with ncp strain of BVDV. Previous studies demonstrated that cp BVDV induces type one interferon (type 1 IFN), whereas ncp BVDV fail to induce type 1 INF **(Adler et al., 1997).** The effect of cp and ncp strains of BVDV is different in *in vivo* than *in vitro* experiments. In contrast to the results of previous *in vitro* studies, experimental infection of calves with ncpBVDV induce strong alpha/beta and gamma interferon responses in gnotobiotic animals **(Charleston et al., 2002).** Type I IFN enhances antibody production both *in vitro* and *in vivo*, including promoting class-switch recombination and polarizing antibody responses toward IgG2 production **(Finkelman et al. 1991)**. This finding is in agreement with the observation of current study where ncpTGAN strain of BVDV showed more IgG2 concentration as compare to ncpBVDV1b-TGAN strain of BVDV at 7, 14 and 35 days p.i.

An in vitro study conducted using ncp Pe515 strain of BVDV showed that ncp BVDV directed the immune response toward TH2 with high levels of B cell growth factor and IL-4 activity with comparatively low levels of IL-2 activity and IFN- gamma **(Rhodes et al. 1999)** while current finding revealed more IgG2 concentration (Th1 immune response) in calves infected with ncpBVDV1b-TGAN strain of BVDV. The difference in result may be due to study design. In previous study, they did not take

paired cp and ncp strains of BVDV to compare difference in biotypes to determine the TH1 or TH2 immune response. The previous study was in *In vitro* while current study was performed *in vivo*. In previous study, they determine the T helper cell polarity on the basis of memory cell proliferation while in current study T helper immune response was measured indirectly via IgG1 and IgG2 concentration in BVDV free calves that were not exposed to BVDV previously.

A study conducted using cp Pe515 and ncp Pe515 strain of BVDV indicated that ncp strain induced a more rapid and superior primary antibody response (TH2) than the homologous cytopathic biotype within 156 days of infection while cp Pe515 showed rapid cellular response (TH1) following challenge **(Lambot et al. 1997)**. The difference in experimental design, the previous study looked the anamnestic immune response while in current study primary immune response was measured. In this study, the antibody concentration was measured within 35 days p.i. where antibody concentration returned to almost the pre-infection levels at 35 days p.i.

From these findings, it can be concluded that both cp and ncp BVDV cause transient humoral immunosuppression. The ncp BVDV1b-TGAN strain of BVDV induced more IgG2 as compare to its homologous cp BVDV1b-TGAC strain of BVDV. More IgG2 concentration indicated that ncp strain of BVDV polarize the immune response toward TH1 (cellular immune response) while cp strain of BVDV polarize the immune response toward TH2 (humoral immune response). To draw any definitive conclusion, the study should be conducted for a longer time with combination the effect

of BVDV on cell proliferation and cytokine production with multiple pairs of cp and ncp BVDVs.

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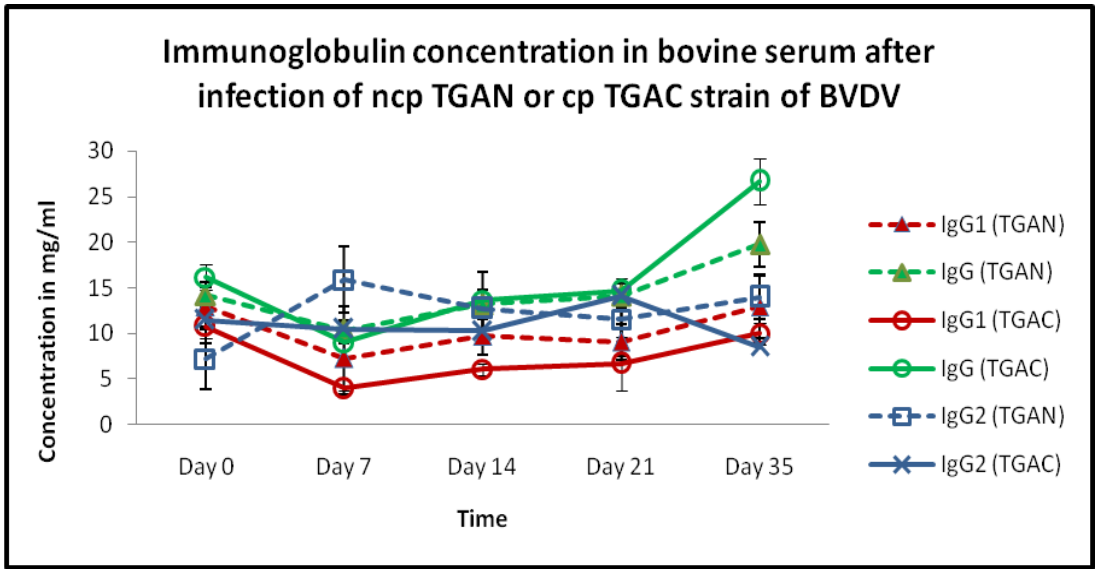

Figure 5-1. The concentration of serum IgG, IgG1 and IgG2 in calves infected with BVDV.

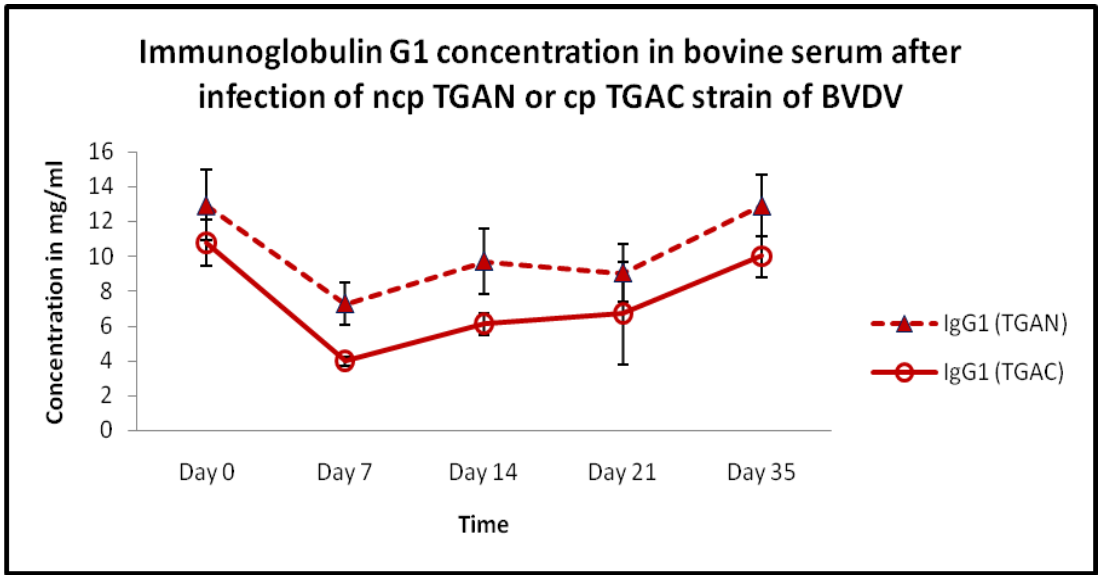

Figure 5-2. The concentration of serum IgG1 in calves infected with BVDV.

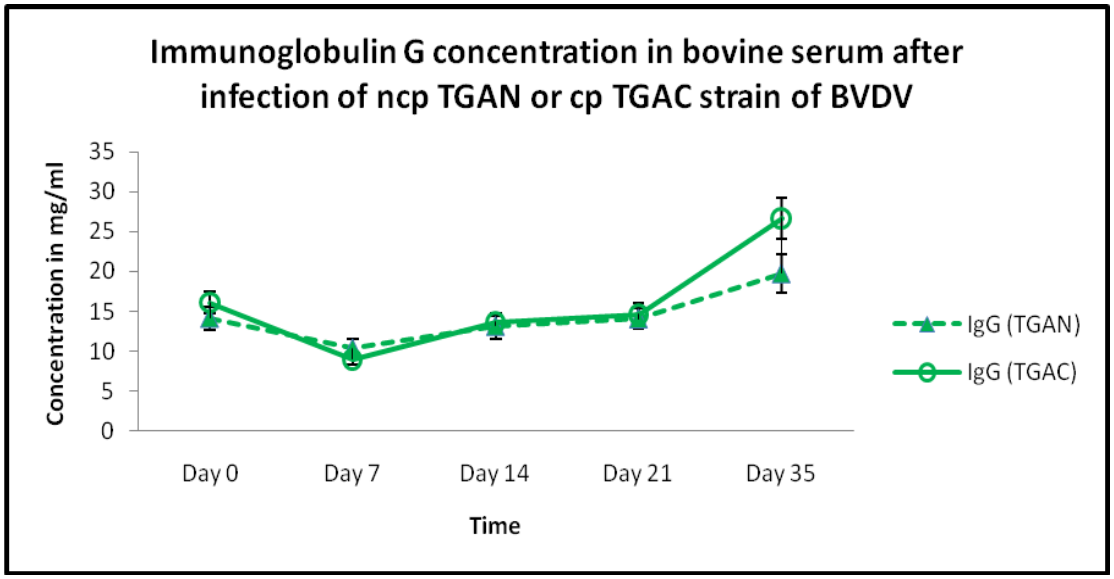

Figure 5-3. The concentration of total serum IgG in calves infected with BVDV.

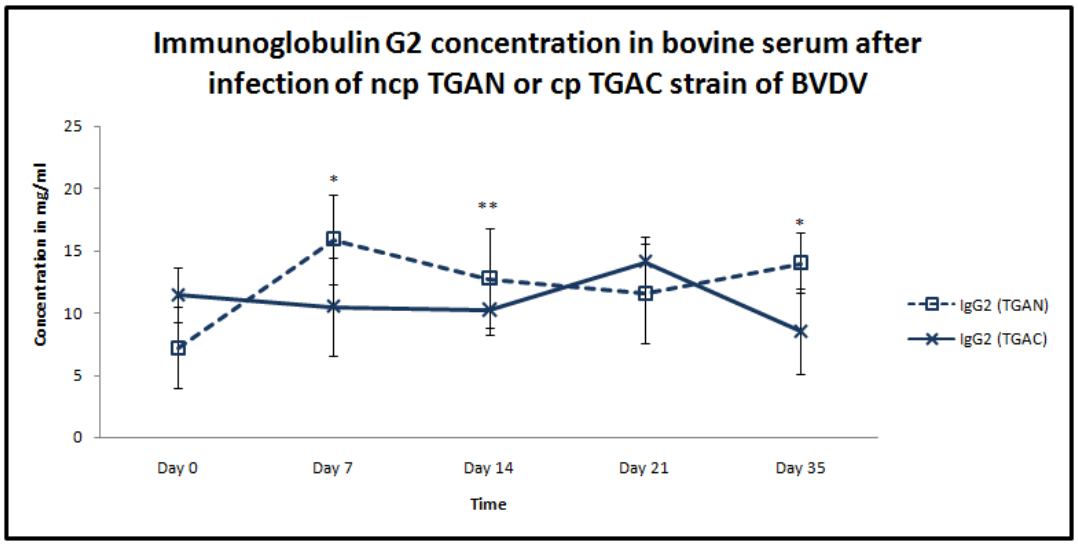

Figure 5-4. The concentration of serum IgG2 in calves infected with BVDV.(The asterix sign showing the significant difference in IgG2 concentrations affected by either cp BVDV1b-TGAC or ncp BVDV1b-TGAN (* p>0.1, ** p>0.05)

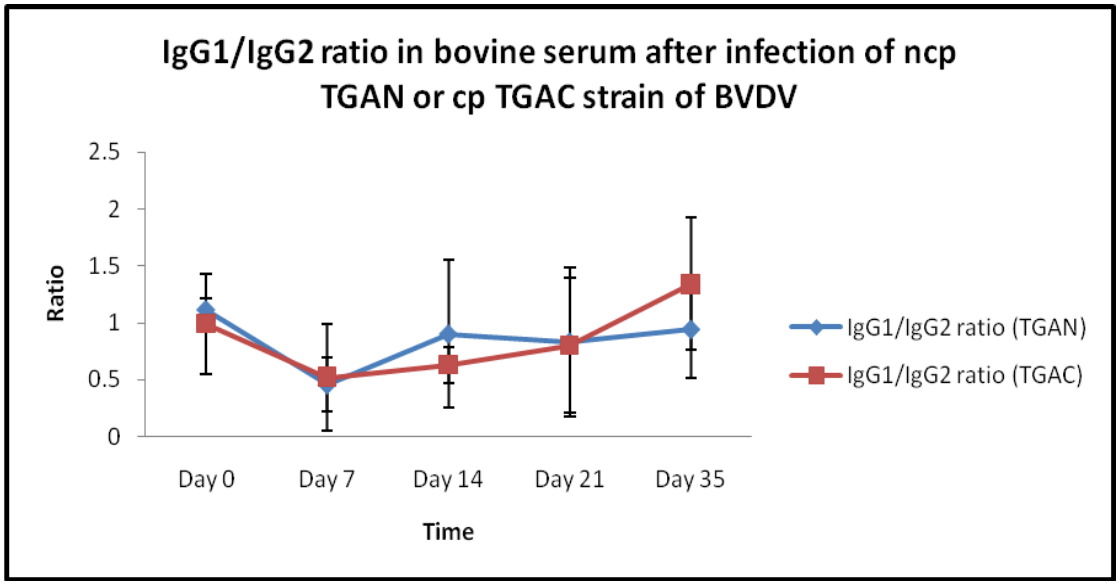

Figure 5-5. The serum IgG1/IgG2 ratio in calves infected with BVDV.

Days of infection	IgG (mg/ml)	IgG1 (mg/ml)	IgG2 (mg/ml)
0 day	16.14±1.46	10.75±1.3	11.44±2.17
7 days	9.00±0.76**	3.95± 0.27**	10.48±3.94**
14 days	13.62±0.87	6.08±0.60	10.32±2.02
21 days	14.68±1.38	6.68±2.94	14.09±2.00**
35 days	26.66±2.56**	9.98±1.18*	8.50±3.39

Table 5-1. The concentration of serum IgG, IgG1 and IgG2 in calves infected with cp BVDV1b-TGAC strain of BVDV. (The asterix sign showing the significant change in concentration of immunoglobulin * p>0.1, ** p>0.05)

Days of infection	IgG (mg/ml)	IgG1 (mg/ml)	IgG2 (mg/ml)
0 day	14.17±1.44	12.93±2.0	7.20±3.27
7 days	10.35±1.11**	7.26±1.18**	15.88±3.59**
14 days	13.17±1.58	9.71±1.88	12.75±4.00
21 days	14.09±1.33	9.05±1.66	11.54±4.03
35 days	19.78±2.43	12.91±1.77	14.00±2.42

Table 5-2. The concentration of serum IgG, IgG1 and IgG2 in calves infected with ncp BVDV1b-TGAN strain of BVDV.(The asterix sign showing the significant change in concentration of immunoglobulin, ** $p>0.05$).

Days of infection	IgG1/IgG2 ratio (TGAN)	IgG1/IgG2 ratio (TGAC)
0 day	1.11±0.11	0.99±0.44
7 days	0.46±0.24	0.52±0.47
14 days	0.90±0.65	0.63±0.16
21 days	0.83±0.65	0.80±0.59
35 days	0.94±0.43	1.34±0.58

Table 5-3. The ratio of IgG1 and IgG2 concentrations in calves's serum infected with either ncp BVDV1b-TGAN or cp BVDV1b-TGAC strain of BVDV

CHAPTER 6

GENERAL DISCUSSION

Antigen presenting cells (APC) play a key role in mounting the immune response. Dendrite cells (DC) are unique APC that make a critical link between the innate and adaptive immune response **(Colino and Snapper, 2003)**. DC activate naïve T cell and produce pro- and anti-inflammatory cytokines. The DC do active surveillance for the antigens. The immature DC are highly phagocytic cells that capture, process and present antigen to T cell in secondary lymphoid organs. The tissue-resident DC migrate from peripheral sites to lymphoid organs to initiate the adaptive immune response and for the maintenance of peripheral tolerance. DC infected with virus may have altered phenotypes and functions that may affect the development of effective immune response. The previous studies with hepatitis C virus (HCV) have shown that virus infected DC have impaired cell surface marker expression. The impaired cell surface marker expression reduced DC maturation and T cell activation **(Saito et al., 2008; Shen et al., 2010)**.

In the current study, we had two DC hypothesis: 1) BVDV-infected DC could serve as a site for virus replication and play a role in virus dissemination 2) DC infected with BVDV have impaired cell surface marker expression associated with antigen presentation (MHCI and II) and co-stimulaiton signals (CD86). If the expression of these surface makers was impaired, T cells activation would be suppressed and this could contribute to immunosuppression . Regardless of the virulence of the strain, BVDV infects the host through the oronasal route. The primary viral replication occurs in nasal

mucosa, tonsils and their leukocytes. The virus spreads from the nasal cavity to regional lymph nodes through lymphatics and blood vessels. In regional lymph nodes, the virus infects the lymphocytes, monocytes and monocyte-derived cells (i.e. macrophages and dendritic cells) **(Brodersen and Kelling, 1998; Bruschke et al., 1998; Kelling et al., 2002)**. The antigen-presenting cells (APC) (dendritic cells (DC), monocytes, and macrophages) and lymphocytes (T and B) are the main target for BVDV **(Bruschke et al., 1998; Liebler-Tenorio et al., 2003)**.

The major objective of this study was to investigate the effect of BVDV infection on DC's surface marker expression and its ability to produce infectious BVDV. Due to DC's anatomical location in skin, mucosa and lymphoid tissue, it is difficult to isolate DC without affecting their cell surface markers and functions. In the current study, an *in vitro* culture system was used to differentiate monocytes into monocyte-derived dendritic cells (MDDC) in large quantity **(Mwangi et al., 2005)**. During differentiation of MDDC, a breed difference was found as monocytes isolated from Brown Swiss (6 of 6; 100%) differentiated better than Holstein Friesian (1 of 18; 5.5%). The MDDC were morphologically and phenotypically similar to classical DC. The MDDC were used as model to classical DC in the *in vitro* study.

A third hypothesis was that BVDV induced cell autophagosomes. BVDV infects almost almost all types of cells. Since it can infect most cells, any effect on the autophagosome system may be important for cellular function, antigen presentation

and/or innate immunity. BVDV may induce the autophagy in infected cells and utilize autophagocytic components for its robust replication as it has been seen with other flaviviruses **(Ait-Goughoulte et al., 2008; Dreux et al., 2009a; Heaton and Randall, 2010; Li et al., 2011; Panyasrivanit et al., 2009; Tang et al., 2012)**. This dissertation investigated the effect of autophagy in BVDV replication

The fourth hypothesis was that the BVDV cause transient humoral immunosuppression in primary infection. The cp biotype of BVDV polarize the immune response toward T helper -2 (TH-2: humoral immune response) while ncp biotype of BVDV polarize the immune response toward T helper -1 (TH-1: cellular immune response). BVDV affect the number of circulating as well as follicular B lymphocytes. The number of B lymphocytes directly affects the production of antibody. Along with this different BVDV biotypes have different effect of cell cultures as well in *in vivo*. Previous studies has shown that the cp BVDV induces type one interferon (type 1 IFN), whereas ncp BVDV fail to induce type 1 IFN in *in vitro* experiments in contrast to that experimental infection of calves with ncpBVDV induced strong type 1 IFN and type 2 IFN**.** Type I IFN enhances antibody production both *in vitro* and *in vivo*, and promotes class-switch recombination and polarizing antibody responses toward IgG2 production. The current study investigated the effect of primary BVDV infection on humoral response and differential effect of ncp and cp BVDV strains on T helper -2 (TH-2) and T helper -1 (TH-1) immune polarity indirectly through IgG1 and IgG2 concentration using

a homologous pair of ncp and cp type 1b viruses Tifton Georgia non-cytopathic (TGAN) or Tifton Georgia cytopathic virus (TGAC) recovered from an animal that died of mucosal disease.

To summarize, the hypotheses were: 1) Infection of monocyte-derived dendritic cells (MDDC) with BVDV causes changes in the phenotype of MDDC (cell surface molecules expression); 2) MDDC are targets of BVDV production resulting in dissemination of BVDV to secondary lymphoid tissues and other tissues; 3) BVDV infection induces autophagy and uses autophagosome for its replication and productive infection; 4) *In vivo* antibody response against cytopathic BVDV polarizes the T cell response to TH2 response while the antibody response to noncytopathic BVDV is polarized to TH1 response

In the current study, the cp TGAC strain of BVDV enhanced MHCI and MHCII expression during course of infection. While the BVDV ncp strains including sever acute 1373, mild acute 28508-5 and TGAN, reduced the MHCI and MHCII expression during the course of infection from 1 hr p.i. to 12 p.i. and from 24 hrs p.i. to 72 hrs p.i. Among the ncp strains of BVDV used in the study, 1373 strain reduced the MHCI and MHCII most followed by 28508-5 and TGAN strain of BVDV. The cp strain of BVDV significantly enhanced the CD86 expression as early as 1 hr p.i. while all ncp strains of BVDV down regulated the CD86 expression through course of experiment. Similarly, a decreased expression of MHC class II was observed in monocytes and monocyte-derived

macrophage (MDM) infected with ncp and cp BVDV **(Chase et al., 2004)** Additionally, decreased expression of MHC class I in monocytes and MDM infected by ncp strain of BVDV and an increase in the case of cp strain of BVDV was observed **(Archambault et al., 2000; Chase et al., 2004)**. The MHCI and MHCII expression is directly related to T cell activation. Reduced T cell proliferation was observed in ncp BVDV infection as compare to cp BVDV infection **(Brackenbury et al., 2003; Collen and Morrison, 2000)**.

The current study revealed the significant up regulation of MHCI, MHCII and CD86 expression in MDDC infected with cpTGAC strain of BVDV while MHCI, MHCII and CD86 expression was reduced in MDDC infected with ncp strains of BVDV. The increased expression of MHCI in MDDC infected with cp BVDV and reduced expression in MDDC infected with ncp BVDV may be due to influence of type 1 IFN. The effect of BVDV on type 1 IFN production varied greatly between *in vitro* experiments (laboratory experiment) and *in vivo* experiments (field experiment).*In vitro* infection of bovine cells with cp BVDV resulted in the induction of type one interferon (IFN-alpha/beta) while most ncp BVDV isolates have not been shown to induce type 1 interferon **(Diderholm and Dinter, 1966)**. Type one IFN up regulates MHCI expression **(Tovey et al., 1996)**. *In vivo* acute infection of naive cattle with ncp BVDV resulted in IFN-alpha/beta production **(Brackenbury et al., 2005)**. To better understand the effect of cp and ncp BVDV infection on APC surface marker expression, an *in vivo* trial should

be carried out. The APC like DC or its precursor (eg. monocytes) should be collected from animals either infected with cp or ncp BVDV. The DC are present in small quantity in circulating blood. These DC population can be isolated from experimentally infected animals by enrichment of cells displaying receptors for plasma fibronectin, followed by adherence and separation on Metrizamide as described earlier **(Renjifo et al., 1997)** or by magnetic microbeads labeled for antibodies against DC markers **(Szczotka et al., 2012)** or by Fluorescence activated cell sorting **(Stasiolek et al., 2012).** The collected APC should be examined for up and/or down regulation of cell surface marker expression with proper controls. The Change in cell surface marker expression can also be determined in DC in fixed tissue through IHC (Immunohistochemistry) and cell surface marker expression can be measured on the basis of intensity of expression in fixed tissue as used earlier **(Hughes et al., 2004)**.

It will be interesting to know how BVDV regulates the MHC molecules and which viral protein(s) is/are responsible for regulation. The MHC I molecules assembles with antigenic peptides in the endoplasmic reticulum (ER) and are transported to the cell surface. At the cell surface, MHC I-peptide complexes interact with cytotoxic T cells (CTL) for their proper activation **(Townsend and Bodmer, 1989)**. These antigenic peptides are generated and processed in the cytosol and are translocated into the ER by the transporter associated antigen processing protein (TAP). The TAP is composed of two polypeptide chains, TAP1 and TAP2 **(Ritz and Seliger, 2001)**. To escape the immune response, viruses have developed various strategies to subvert the antigen

processing and presentation. The HCV core protein enhances the MHCI expression via p53-dependent upregulation of TAP1. Upregulation of MHCI expression resulted in impaired NK cell (natural killer cell) cytotoxicity **(Herzer et al., 2003)**. Several viruses down regulate MHCI. Herpes simplex virus (HSV) downregulates the MHCI molecule expression by retaining the MHC I proteins in the endoplasmic reticulum (ER), possibly through interference with the TAP (**Rosenberg, 1999)**. Similarly the adenoviral glycoprotein E3/19K down-regulate cell surface MHC I expression by retention of MHC H chains in the ER **(Andersson et al., 1985; Burgert and Kvist, 1985)**. The Nef protein of HIV (Human immunodeficiency virus**)** down-regulates the MHC I expression via enhanced MHCI endocytosis by the PI3K-regulated ARF6 pathway **(Blagoveshchenskaya et al., 2002; Piguet et al., 2000)**. The human pathogen Kaposi's sarcoma-associated herpesvirus encodes two gene products, K3 and K5. The K3 and K5 reduce the number of MHC class I molecules at the cell surface by facilitating their endocytosis **(Ishido et al., 2000)**. Along with this the K3 protein targets the MHCI for lysosomal degradation following endocytosis **(Hewitt et al., 2002).**

One of the more interesting approaches to understand how BVDV down regulates the MHC molecules would be to analyze the viral protein(s) that is/are responsible for down regulation. Our lab has cloned various structural and non-structural proteins of BVDV. To evaluate the effect of different viral proteins on MHC molecule expression in MDDC, the MDDC should be transfected with each viral protein separately and should be examined for MHC molecule expression. The only limitation in this study is that, we

are not able to transfecte the MDDC so far while bovine endothelial cell is transfected well with these proteins. The endothelia cells express high amount of MHCI molecules. The effect of BVDV viral proteins on MHCI expression can be measured in BVDV protein transfected bovine endothelial cells. To determine the strain effect of ncp or cp BVDV, the bovine endothelial cells can be transfected either with NS23 protein to measure the effect of ncp BVDV or with NS2 or NS3 or both to measure the effect of ncp BVDV on MHCI expression.

The major difference between cp and ncp biotypes of BVDV is that ncp BVDV has uncleaved NS23, whereas this protein is cleaved into NS2 and NS3 in cells infected with the cp biotype of BVDV **(Brownlie, 1990)**. The NS2 protein, in conjunction with the amino terminus of NS3, functions as an autoprotease that cleaves the NS2-NS3 junction of the polyprotein **(Kummerer and Meyers, 2000; Lackner et al., 2004)** NS3 acts as serine protease **(Tautz et al., 1997; Wiskerchen and Collett, 1991; Xu et al., 1997)**, NTPase **(Tamura et al., 1993)** and helicase **(Warrener and Collett, 1995)**. These functions are essential in pestiviral RNA replication which cannot be supplied by its NS23 precursor **(Lackner et al., 2004)**. Because of the multifactorial effect of NS3 protein, it will be interesting to know the role of NS3 protein in regulation of MHCI, MHCII or CD86 molecules.

In the second study, we determined that MDDC do not support the production of infectious BVDV. The progenitor cells of the MDDC, the monocytes, became infected with BVDV and produced infectious virus.

For MDDC, bovine herpesvirus 1 (BHV1) was use as a positive control virus while the MDBK cells were used as permissive cell control. BHV-1 productively infects many different cell types. These cells include monocytes and macrophages **(Forman et al., 1982; Nyaga and McKercher, 1979)**. MDBK cells produced infectious BVDV as early as 12 hrs p.i. Both MDDC and MDBK cells produced infectious BHV1.The MDDC produced infectious BHV1 at 12 hrs p.i. and the maximum viral titer was reached within 48 hrs p.i. These results were in sharp contrast to another study that showed that bovine DC did not support BHV1 replication **(Renjifo et al., 1999)**. The major difference in these results may be due to differences in DC population used in the studies. In previous study, two DC populations were isolated from peripheral blood by physical means while in current study, MDDC were differentiated from monocyte in the presence of GMCSF and IL-4 and characterized phenotypically and morphologically to be dendritic cell. MDDC do not support BVDV production while their progenitor cells, the monocytes, became infected and produced infectious BVDV. To investigate at which stage of differentiation MDDC lose the ability of to produce infectious BVDV, the monocytes were cultured for 1, 2, 3, 4, or 5 days with GMCSF and IL-4 and then infected with BVDV. The intermediate stages of monocytes-MDDC were collected at different time points and infected with BVDV. We found that MDDC lost the ability to produce infectious BVDV at 120 hrs of differentiation.

Since MDDC were unable to produce infectious BVDV, where did the block in virus production occur? To investigate this question, the MDDC were infected with

different strains of BVDV and viral RNA was extracted at different time points. The viral RNA was quantified using RT-PCR. We found that the viral RNA replicated in MDDC. The kinetics of viral RNA production was different between different viral stains. For negative control, the MDDC infected with UV inactivated 1373 strain of BVDV neither produced infection virus nor replicated the viral RNA, This control experiment eliminate the chance of residual viral RNA contamination in MDDC that can be measured by highly sensitive qRTPCR assay and confirmed the MDDC are susceptible for infectious BVDV.

The western blot using MDDC lysate infected with 1373 strain of BVDV revealed the presence of NS5A. The presence of viral protein indicated that viral proteins are translated in MDDC. The BVDV viral RNA is translated into polyprotein. The viral polyprotein is cleaved into 10-11 different structural and non-structural viral proteins including NS5B either by viral or cellular protease **(Donis, 1995; Harada et al., 2000)**. The NS5B act as a RNA dependent RNA polymerase and replicates the viral genome. The presence of viral RNA and viral proteins in MDDC indicated that BVDV infects MDDC and replicates the viral RNA and translates the viral protein but does not release infectious virus. The inability to release infectious virus from MDDC may be due to hindrance in viral assembly or release.

At this stage we cannot rule out the different possibilities that may block the production of infectious BVDV. Inability to produce infectious BVDV by MDDC may be due to insufficient synthesis of viral proteins or instability of one or more viral

proteins as was observed in influenza A virus in Vero cells **(Lau and Scholtissek, 1995)**. Additional studies needs to be done to quantify the viral proteins in MDDC and determine their stability.

The presence or absence of particular host factors in MDDC may also restrict the viral assembly. Studies with parainfluenza virus 5 have shown that the regulatory protein 14-3-3 can inhibit viral assembly by sequestering M protein, thereby preventing its incorporation into virions **(Pei et al., 2011)**. BVDV is an enveloped virus and released through budding. It is prerequisite that viral glycoprotein should integration into the host cell membrane before budding. Studies have shown that BVDV glycoproteins (Erns and E2) aew integrated to ER membrane prior to release into its cisternae by budding **(Grummer et al., 2001)**. To confirm the proper assembly of BVDV glycoproteins (Erns and E2) into ER membrane, a co-localization study should be done in MDDC with the proper cell control.

In third study both ncp and cp strains of BVDV induced autophagy. The autophagy induction in BVDV infected cells was significantly different than non-infected cells. We could not find any significant difference between cp and ncp strain of BVDV in formation of autophagosomes. The autophagy inducing drug, rapamycin, increased the autophagy and BVDV replication while autophagy inhibiting drug, 3MA suppressed the autophagy and BVDV replication. The co-localization study using BVDV NS5A and BVDV E1 proteins with autophagosomes marker LC3 revealed that BVDV did not replicate in autophagosomes while the increase of autophagosomes facilitated the

replication of BVDV in infected cells. These results indicated that the autophagy machinery supported viral RNA replication by possibility providing energy or factors required for translation of incoming hepatitis C virus **(Dreux et al., 2009).** There have been three major autophagosome induction pathways that have been well studied. The first pathway involves the mammalian target of rapamycin (mTOR). The mTOR negatively regulates autophagy **(Jung et al., 2010; Yang and Klionsky, 2010)**. The second pathway is mediated by Atg6/Beclin1 **(Furuya et al., 2005)**.The third is regulated by two ubiquitin-like molecules, Atg12 and LC3/Atg8 **(Fujita et al., 2008)**. The Atg12 and LC3/Atg8 molecules are involved in autophagosome biogenesis. Until now, none of these pathways have been studied for autophagy induction by BVDV. Further study needs to be carried out to determine whether any one of the above mechanism or combination of them could be responsible for autophagy induction in BVDV infected cells.

Autophagy pathways have been characterized in other flaviviruses. The NS4B protein of HCV induces autophagy via interactions with both the early endosome-associated GTPase Rab5 and a class III phosphoinositide 3-kinase, Vps34 **(Su et al., 2011)**.The Dengue-2 and Modoc (a murine flavivirus) infection induced the PI3K-dependent autophagy in MDCK cells. The PI3K-dependent autophagy was mediated by flavivirus NS4A gene. A further study is needed to determine which BVDV protein(s) is/ are responsible for induction of autophagy by tranfecting the bovine endothelia cells with

different BVDV protein(s) and measuring the gene expression of either mTOR or Beclin1 or Atg12 or LC3/Atg8 or all.

In the fourth study, both cp TGAC and ncp TGAN strains of BVDV caused humoral immunosuppression by reducing total serum IgG and the IgG1 concentration (reduced Th2 or humoral immune response). The immunosuppression was transient and was restored by 35 days p.i. The ncp TGAN strain of BVDV enhanced the IgG2 production while cp BVDV strain enhanced the IgG1 production through the study. These result indicated that ncp strain polarized the immune response toward T-helper 1 (cellular immune response) while the cp strain of BVDV polarized the immune response toward T- helper 2 (humoral immune response).

In this study the fourteen BVDV seronegative calves were divided in to two groups with seven calves each. One group was infected with ncp TGAN strain of BVDV while another group was infected with homologous cp TGAC strain of BVDV intranasally. The blood was collected and serum was separated to measure the concentration of total IgG, IgG1 and IgG2 at at 0, 7, 14, 21 and 35 days p.i. Both cp and ncp BVDV reduced the total IgG concentration to two third amounts than the initial within 7 days p.i. The total IgG concentration gradually increased from 7 days p.i. to 35 days p.i. The total IgG concentration increased approximately 1.6 times at 35 days p.i than initial concentration in calves infected with cp TGAC strain of BVDV while it increased around 1.3 times than initial concentration in calves infected with ncp TGAN strain of BVDV at 35 days p.i.

A similar pattern was observed in serum IgG1 in calves infected with cp TGAC or ncp TGAN. Both cp and ncp BVDV caused transient humoral immunosuppression. To draw any definitive conclusions, the study should be conducted for longer period of time with combination of the findings of cell proliferation and cytokine production following BVDV infection. The multiple pairs of cp and ncp BVDV should be included in the study for strengthen the results.

In summary, there are several key questions that emerged from these studies:

The ncp strains of BVDV down regulated the MHCI, MHCII and CD86 expression on MDDC while cp BVDV upregulated the MHCI, MHCII and CD86 expression. The major difference in cp and ncp strain of BVDV is cleavage of NS23 protein. The NS23 protein is not cleaved in ncp BVDV while it is cleaved into NS2 and NS3 in cp BVDV. Is NS2 or NS3 or other BVDV viral proteins having an effect in regulation of MHC molecules? Our lab has cloned the NS2 and NS3 protein and other BVDV proteins. It will be worth interesting to transfect the MDDC with each of these proteins separately and evaluate their effect on MHC molecule expression.

1) Is the increased MHCI module expression in cp BVDV infection and down regulation in ncp BVDV infection due to the effect of type one interferon? To answer this question the MDDC infected with either cp or ncp BVDV should be examined for expression of type 1 IFN gene. The supernatant of infected cells could also be quantified for type one IFN release. To further confirm the effect of

type one IFN on cell surface expression, the ncp BVDV infected MDDC should be supplemented with type one IFN and examined for MHCI expression.

2) Since it is well known that MHCI and MHCII expression affect T cell activation and BVDV affects MHCI and MHCII expression the obvious question is-what effect does BVDV infection of MDDC have on T cell activation? To answer this question, MDDC should be infected with BVDV and used as APC to T helper cells. MDDC cells should be "infected" with BVDV neutralized with BVDV antibody. The antibody neutralized BVDV would enter the MDDC through antibody dependent endocytosis and process and present antigen through MHCII. The effect of BVDV on T helper cell activation could be measured using this procedure.
3) In this study, MDDC did not support BVDV production. The progenitor cell of the MDDC, the monocyte, became infected and produced infectious BVDV. As monocytes differentiated to MDDC, they lost virus production capacity at 120 hrs of differentiation. The presence of viral RNA and viral proteins in MDDC indicated that BVDV replicate and translate viral proteins in MDDC but do not release infectious virus. The inability to release infectious virus from MDDC may be due to hindrance in viral assembly or release. The next questions that arise are what are the factors that prevent either assembly or release of virus? There may be presence or absence of particular host factors in MDDC that restrict viral assembly or release. In the next experiment a protein profile comparison could be

done between viral permissive cells (like MDBK cells, Bt cells) and non-permissive cells (MDDCs and MDM) to determine the preliminary difference. The BVDV structural viral protein ((Erns and E2 glycoproteins) assembly occur at ER membrane and immature virus is released in to into cisternae of ER by budding. To know whether there is hindrance in viral assembly, a co-localization study using BVDV viral proteins and ER should be done in MDDC. There may be insufficient synthesis of viral proteins or instability of one or more viral proteins that result in abortive infection of BVDV in MDDC as it was seen for influenza virus and mouse dendritic cells **(Ioannidis et al., 2012)** A further study needs to be done to quantify the viral proteins in MDDC and their stability in MDDCs at different time points with proper control.

4) In this study determined that both ncp and cp strains of BVDV induced autophagy. The next question that arises is which viral factor(s) (structural or nonstructural proteins) induce autophagy? Secondily, what pathways are used by BVDV to induce autophagy? To answer the first question, the cell could be tranfected with different viral proteins separatly and examined for autophagy induction. To answer the later question the all three well known pathways a) the mammalian target of rapamycin (mTOR) pathway, b) Atg6/Beclin1 pathway, and c) Atg12 and LC3/Atg8 pathway should be examined after BVDV infection and corelated with induction of autophagy.

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